"This is because one does not think without becoming something else, something that does not think – an animal, a molecule, a particle… "

"Every sensation is a question, even if the only answer is silence"

-Gilles Deleuze and Felix Guattari, What is Philosophy?

"I see from one point, but in my existence I am looked at from all sides"

-Jacques Lacan, Four Fundamentals of Psychoanalysis

"Nature loves to hide"

-Martin Heidegger quoting Heraclitus

" … la patience scientifique. Sans cet interet, cette patience serait souffrance. Avec cet interet, cette patience est un vie spirituelle" [… scientific patience. Without this interest, this patience would be suffering. With this interest, this patience is a spiritual life.]

-Gaston Bachelard, La Formation de l'Esprit Scientifique

"Wo es war, soll ich werden" [Where it was, I shall come into being]

- Sigmund Freud

"... the seeker after knowledge forces his spirit to recognize things against the inclination of the spirit, and often enough also against the wishes of his heart... and thus acts as an artist... of cruelty."

-Friedrich Nietzsche, Beyond Good and Evil, p.159

University of Alberta

Description of CD8α on Human Monocytes and Its Enhancement of FcγR Responses

by

Derrick Gibbings ©

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy in Experimental Medicine

Department of Medicine

Edmonton, Alberta
Fall 2006

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ABSTRACT

CD8 is a transmembrane dimeric glycoprotein (CD8αα or CD8αβ) expressed by a subpopulation of cytotoxic T cells (CTL). Responses of CTL stimulated through the T cell receptor (TCR) can be enhanced by CD8 binding to MHC class I.

In contrast to mouse NK cells and macrophages, human and rat NK cells and rat macrophages express CD8. The expression of CD8 by human macrophages or monocytes had not been adequately examined. I present strong evidence that human monocytes express CD8α, but not CD8β.

O-glycosylation and palmitoylation of CD8 affects its ability to co-stimulate CTL through TCR. I found that CD8 on monocytes can be differentiated from CD8 on T cells by binding of a single anti-CD8α mAb, and by 2-D electrophoresis. Some of these differences between CD8α on monocytes and T cells are independent of sialylation, suggesting a novel difference in CD8α between cell types.

A motif in macrophage migration inhibitory factor (MIF) resembling MHC class I was serendipitously discovered while studying the binding of CD8 to rat MHC class I. MIF similarity to MHC class I was defined by sequence analysis and binding of anti-MHC antibodies to MIF and MIF-derived peptides. MIF weakly inhibited binding of MHC class I to NK cells. Some activities of MIF such as inhibition of NK cell cytotoxicity and chemotaxis may be accounted for by MIF binding to select receptors for MHC class I.

CD8 can enhance inflammatory responses or induce apoptosis of NK cells and macrophages. However, how these effects were regulated, since the CD8 ligand, MHC class I,

is ubiquitously expressed by most cells was unknown. I suggest CD8 may be regulated by and co-stimulate cells through receptors that use signaling proteins similar to TCR, like linker for activation of T cells (LAT). FcγR is a family of receptors, found on human monocytes and other cells that uses similar signaling mechanisms to TCR. CD8 enhanced monocyte TNF release and signaling through LAT, when monocytes were co-stimulated with immune-complexes, likely in an FcγR-dependent manner. This evidence predicts for the first time that CD8 can enhance responses of receptors other than TCR, like FcγR.

DEDICATION

To all those people who had the patience.

To the terrifying spaces and screaming wind of Saskatchewan

For driving me to this.

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Appendix 2

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LIST OF ABBREVIATIONS

ADCC, Antibody-Dependent Cell-mediated Cytotoxicity

β2m, β2-microglobulin

B-CLL, B cell chronic lymphocytic leukemia

CEA, Carcinoembryonic Antigen

CTL, Cytotoxic T cells

CRTAM, Class I-Restricted T cell-Associated Molecule

EAE, Experimental Allergic Encephalomyelitis

EPCR, Endothelial Receptor for Protein C

FAK, Focal Adhesion Kinase

FcR, Fc Region of Immunoglobulin-Receptor

FRET, Fluorescence Resonance Energy Transfer

Hsp: Heat shock protein

IEL, Intra-epithelial Lymphocytes

Ii: invariant chain

ILT, Immunoglobulin-Like Transcript

ITIM, Immune Receptor Tyrosine Based Inhibitory Motifs

JAB1: c-Jun activation domain binding protein-1

JNK: c-Jun N-terminal kinase

LAK: Lymphokine Activated Killer Cell

LAT, Linker for Activation of T cells

LILRB1: Leukocyte immunoglobulin-like receptor subfamily B member 1

MIC-A, MIC-B, MHC class I Polypeptide Related Sequence

MIF, Macrophage Migration Inhibitory Factor

MAb, Monoclonal Antibody

MLCK, Myosin Light Chain Kinase

NKG2D, NK Cell Receptor D

NKRP, NK Cell Receptor Protein

OPD: o-Phenylenediamine dihydrochloride

PI3K, Phosphatidyl Inositol-3 Kinase

PIRB: Paired-immunoglobulin-like receptor-B

PLCγ, Phospholipase Cγ

Rae1β, Retinoic Acid Early Inducible Protein 1

RCMC: rat cultured mast cell line

SPR, Surface Plasmon Resonance

TCR, T Cell Receptor

TCRα-cpm, TCRα-Connecting Peptide Motif

TIM: triose phosphate isomerase

TLR, Toll-Like Receptor

UL18, Unique Long Region Protein 18

VAST, Vector Alignment Search Tool

ZAG, Zn-α-2-Glycoprotein

LIST OF SYMBOLS

α, alpha

β, beta

δ, delta

ε, epsilon

γ, gamma

η, eta

ζ, zeta

1. INTRODUCTION

1.1. Prologue:

Most of the studies in this thesis are composed of a characterization of the expression, properties and function of CD8 on human monocytes. A vast body of evidence spanning nearly three decades has characterized the structure and modifications of CD8 on T cells and determined that CD8 has an important influence on T cell development and activation of T cell-mediated cytotoxicity through the TCR. Convincing evidence demonstrates CD8 is expressed by mouse dendritic cells, rat macrophages and rat and human NK cells, but which cell types express CD8 depends on whether rat, mouse or human species are examined. Mouse monocytes do not express CD8, but the possibility that human monocytes expressed CD8 had not been rigorously tested.

With an important role in activation of T cells and potentially other cells CD8 might be subject to regulation through expression, physical modification, or inter-molecular interactions. Differences in the addition of sialic acid to O-glycosyl structures on CD8, and CD8 palmitoylation have been previously shown to regulate the ability of CD8 to activate T cells. Experiments here suggest by two means that CD8 expressed by monocytes can be differentiated from CD8 expressed by T cells, in ways unrelated to sialic acid. Modifications of CD8 specific to monocytes may allow the adaptation of CD8 to its situation on monocytes, and hint at a wider heterogeneity of CD8 than currently appreciated.

Many of the functions of CD8 in signaling and cell activation are thought to be limited to T cells, making it difficult to imagine what the function of CD8 on monocytes, for example, might be. Accordingly, minimal data has been published on CD8's signaling, ligand-binding, and potential co-operation with receptors on dendritic cells, NK cells and monocytes. In this thesis I have performed studies to demonstrate the expression of CD8 on human monocytes, and to elucidate if and how CD8 may co-operate with other receptors, like FcR, to activate monocytes. Data presented here suggests new models of CD8 expression, molecular heterogeneity, and co-operation with partner receptors.

Finally, in studying CD8 interaction with MHC class I, novel observations were made that suggest that a small motif that resembles MHC class I may be found in macrophage migration inhibitory factor (MIF). An MHC class I-like motif and function could account for several functions previously attributed to MIF, and present an example of the complexity that emerges when multiple functions are associated with separate regions of one protein.

1.2. Overview of CD8 Function in Cytotoxic T cells

To approach questions of the expression and function of CD8 on monocytes, the primary topic of this thesis, it will be necessary to first survey relevant aspects of CD8 learned from its role in activation of cytotoxic T cells (CTL) through the T cell receptor (TCR).

CD8 is a dimeric[1] glycoprotein. Each CD8 monomer, CD8α or CD8β consists of an Ig –V like domain, an extended O-glycosylated stalk region, and a single-pass transmembrane domain that extends into a cytoplasmic region (Figure 1-1). In *ex vivo* cells dimers of CD8αα and CD8αβ, but not CD8ββ have been described [1].

---

[1] The two subunits of CD8, CD8α and CD8β are separately transcribed and translated from different genes [201]. CD8, to current knowledge is always expressed as a dimer, of CD8αα or CD8αβ, and potentially CD8ββ (in human only) [1]. Throughout this thesis I will refer to CD8α, CD8β, CD8αα, CD8αβ, or CD8ββ to designate properties specifically attributed to the aforementioned subunit or dimer combination of CD8. When "CD8" is used as a term this will refer to properties attributable to all forms of CD8, or properties that cannot or have not been differentially ascribed to CD8α, CD8β, or a specific dimer.

The Ig domains of CD8 bind MHC class I at a site distant from the TCR-binding site [2;3]. If CD8 is present TCR binds more MHC class I loaded with agonistic peptides [4]. This may be due to CD8 and TCR binding the same MHC class I-peptide complex as the co-receptor model proposes, or to CD8 promoting interactions of T cells and target cells, or soluble MHC class I that otherwise promote TCR binding to MHC class I.

The intracellular region of CD8 binds to the src kinase lck and may associate with linker for activation of T cells (LAT) [5;6]. CD8α, Lck and LAT dramatically impact T cell development both during positive and negative selection processes [7-10]. More specifically, the interaction of CD8α with lck is important for T cell development as mutated lck mutated that will not bind CD8, or mutated CD8 mutated that will not bind lck severely perturbs T cell positive and negative selection [11;12].

The abilities of CD8 to bind the same ligand as TCR, namely MHC class I, and to initiate intracellular signaling promote T cell activation through TCR (reviewed in [13;14]). CD8 can enhance, sometimes dramatically, CTL cytokine release and cytotoxicity [15-17]. As such, a lack of CD8 aggravates pathology ensuing from infection with intracellular pathogens and tumorogenesis [16;18-21]. However, interactions of high affinity TCR with MHC class I loaded with specific peptides or a high dose of MHC class I-peptide can generate T cell activation without CD8 [22;23]. Thus TCR may bind MHC class I, recruit lck and LAT, and proceed through T cell activation, without CD8. However, *in vivo* most TCR probably have low affinity for MHC-peptide and depend on CD8 for CTL activation (e.g. [24]).

There is more than one stage of T cell activation, at which CD8 may be bound to and potentially recruit lck and LAT (Figure 1-2). CD45 initially dephosphorylates lck (potentially bound to CD8). Lck then phosphorylates two sites on CD3ζ that allows recruitment and activation of ZAP-70 [25]. ZAP-70 phosphorylates LAT (potentially associated with CD8, Figure 1-2, reviewed in [26]). LAT can centre a scaffolding network of intracellular signaling complexes by recruiting Grb2, SLP-76, and phospholipase Cγ (PLCγ) that will activate or strongly influence calcium flux, cytoskeletal re-organization, proliferation and gene transcription.

1.3. Expression of CD8 Among Cell Types

CD8 contributes to T cell activation through several functions including MHC class I binding and signaling through lck and LAT. As will be discussed later, CD8 may perform some or all of these functions on monocytes, macrophages, NK cells and dendritic cells. First, it will be necessary to better understand the different types of cells that can express CD8, because in some cases CD8 protein is adsorbed or retrieved from other sources by cells that do not synthesize CD8. In these cases CD8 may not be functional, and therefore be of little interest besides as a potential source of error in cell typing.

1.3.1. Cell Types That Express CD8 Vary Across Mammalian Species

While CD8 is expressed on a variety of immune cell types in rats and humans, CD8 expression appears to be limited to T cells and dendritic cells in mice. These differences among species in cells that express CD8 may belie broader differences among species in CD8, TCR, or other receptors and their signaling mechanisms. To understand the function of CD8 itself, or CD8+ cells in studies that use either gene knockout models or anti-CD8 antibody *in vivo* to indiscriminately deplete CD8+ cells, it will be necessary to understand differences among species in which cell types express CD8. Depending on the species, CD8 on macrophages, monocytes, dendritic cells and NK cells may contribute to effects in these experiments that are, at times simplistically, attributed to T cells.

CD8 is expressed as dimers of CD8αα and CD8αβ. Human CD8α is required for CD8 homodimers or heterodimers to reach the cell surface [27], with one potential exception raised below [1]. Therefore CD8α expression will be discussed as a surrogate for any CD8 expression in this section. In the following section the different dimer combinations of CD8 on each cell type will be discussed.

1.3.1.1. A Subpopulation of T cells and Dendritic Cells Express CD8α in Rats, Mice and Humans

The expression of CD8 at high levels on a subpopulation of T cells makes it easily detectable. CD8 is also detected on a subpopulation of dendritic cells in rat [28;29], mouse [30], and human [31]. The discovery of the expression of CD8 by dendritic cells was delayed due to their late characterization and the difficulty in procuring these cells. Convincing studies using mice deficient in T cells and transplanted chimeric dendritic cells demonstrated that mouse dendritic cells from spleen and thymus synthesize CD8α, rather than acquire mRNA or protein from phagocytosis of apoptotic T cells, or from the T cell membrane [30].

1.3.1.2. NK Cells Express CD8α only in Rat and Human, Not Mouse

In some species CD8 is expressed on NK cells, monocytes and macrophages; perhaps the low level of CD8 expression on these cells led to its more recent description. Interestingly, current evidence suggests CD8α protein is detected on NK cells in rat [32;33] and human [34-37], but not in mouse [36] (Table 1-1, Part I). Purified human NK cells re-synthesize CD8 after its proteolytic cleavage from the cell surface [35], but similar studies have not been done in rat. Thus, a careful description of the expression of CD8 on NK cells that would exclude soluble CD8 being adsorbed, bound to MHC class I on NK cells, or acquired with membrane of CD8+ cells rather than being synthesized is lacking to the author's knowledge. This is relevant as NK cells are known to acquire membrane fragments from target cells [38].

1.3.1.3. Rat But Not Mouse Monocytes and Macrophages Appear to Express CD8

Mouse CD8α+ dendritic cells may originate from either lymphoid or myeloid cells [39-42], suggesting both lymphoid and myeloid lineages of progenitors can express CD8. Rat alveolar and peritoneal macrophages and a macrophage cell line contain CD8α and CD8β mRNA, and the respective proteins are present on the cell surface, demonstrating that myeloid cells can produce CD8α themselves, and not only acquire it from other CD8+ cells [43;44]. Rat monocytes are bound by anti-CD8α mAb [45] and $F(ab)_2$ fragments thereof [46] suggesting they have CD8α protein on their surface. It is not certain that rat monocytes synthesize CD8 protein on their surfaces, or acquire it from other cells.

CD8α protein is found at low levels on mouse splenic macrophages, even in T cell deficient mice [30], although macrophages may acquire CD8 from CD8+ dendritic cells in this system. While CD8α mRNA was detected in mouse alveolar macrophages picked as single cells by morphological criteria, CD8α protein was not detected on these cells (Genevieve Nault, thesis). Furthermore, mouse monocytes do not appear to express CD8α [47]. Thus, no convincing evidence demonstrates mouse monocytes or macrophages express CD8α, and the majority of evidence suggests they do not (Table 1-1, Part I).

1.3.1.4. No Conclusive Evidence Exists to Demonstrate Whether Human Monocytes or Macrophages Express CD8

Several groups have found that CD8α mAb bind the surface of human macrophages [48-50], but in the past these studies often suffered from lack of isotype antibody controls, essential when working with Fc region of Immunoglobulin-Receptor (FcR)-+ macrophages, and did not demonstrate that CD8 was synthesized by macrophages. Similarly, expression of high levels of CD8α by a subpopulation of human monocytes was recently described by two laboratories [51;52]. While isotype controls for anti-CD8α mAb were included in one paper in Journal of Medical Virology [52], no isotype control antibodies were shown or discussed for flow cytometry, and no RT-PCR for CD8 mRNA was performed in the other publication in Blood [51].

Unfortunately, thorough studies to establish the synthesis of CD8 including RT-PCR, or pulse-chase analysis and binding of multiple anti-CD8 F(ab)2 monoclonal antibodies have only been performed on rat macrophages (CD8+)[44;53], and mouse monocytes and macrophages (CD8-, Genevieve Nault, unpublished data) [47]. Due to the frequent lack of isotype control antibodies or information about the synthesis of CD8α, it was impossible to draw conclusions about the expression of CD8α by human monocytes and macrophages when the work in this thesis commenced. One of the primary aims of this thesis was to examine whether human monocytes synthesize functional CD8 themselves or acquire non-functional CD8 from secondary sources.

1.3.2. Expression of Dimer Combinations of CD8α and CD8β Varies Among Cell Types and Species

There are differences between rat, mouse, and human in which cell types express CD8α and CD8β (summarized in Table 1-1, part I and II). Perhaps both CD8αα and CD8αβ dimers exist as an adaptation of CD8 functions to specific roles in TCR activation, or activation of other cells. CD8αα is induced on some activated CD4+ and CD8+ T cells prone to develop into memory T cells [54]. CD8αα is also expressed on NK cells [35] and mouse dendritic cells [30]. CD8α and CD8β are expressed by a subpopulation of T cells in rat, mouse and human as well as on T cells in lower vertebrates including fish, birds, and amphibians [55-59]. A consensus has been built that CD8β allows optimal co-operation of CD8 with αβ TCR, largely based on the presumption that CD8αβ dimers are expressed exclusively on αβ TCR T cells [60-65]. Unfortunately, this consensus ignores the fact that CD8αβ is expressed and enhances immune responses on macrophages [44], and mast cells [53] in rats and γδ T cells in mammals and avians [57;66-68]. This suggests CD8αβ is not restricted to functioning through a specific physical interaction with the αβ TCR as has been suggested [63;65;69;70]. The expression of CD8αβ on cells other than αβ TCR T cells has not been thoroughly investigated in humans. Accordingly, we examined whether CD8αβ is found on human monocytes. First understanding which cell types express CD8αα and CD8αβ among species will help elucidate specific and differential roles of CD8αα and CD8αβ in activation of cells.

CD8αβ heterodimers may also be found on some myeloid populations (below). CD8β has been described on CD3-/TCRαβ-/TCRγδ- bone marrow veto cells, that may be NK cells or myeloid cells [71]. CD8β was detected on mouse thymic dendritic cells, albeit at lower levels than CD8α (presumably CD8αβ and CD8αα dimers are present) [72;73]. However mRNA for CD8β was not detected in thymic dendritic cells, and CD8β protein detected on thymic dendritic cells probably derives from T cells [73]. If CD8β is acquired by dendritic cells with a piece of membrane from other cells it could retain binding and signaling functionality on dendritic cells for a few hours.

In contrast to mouse dendritic cells, rat macrophages synthesize CD8β protein. CD8β mRNA and protein was detected in a rat macrophage cell line, free from a source of contaminating CD8 from T cells, NK cells or dendritic cells [53]. *Ex vivo* rat macrophages, monocytes and mast cells have CD8β protein at the plasma membrane and CD8β mRNA [44;51;74]. CD8α was present at higher levels than CD8β on rat macrophages and mast cells [44;74]. Taken together, some rat myeloid cells express CD8α and CD8β, suggesting CD8αβ heterodimers are found alongside CD8αα homodimers on these cells.

When CD8α and CD8β are transfected into CD8- cells more CD8α is detected than CD8β [1;62], suggesting a large proportion of CD8αα dimers are expressed, alongside a limited number of CD8αβ heterodimers. Similarly, studies on *ex vivo* T cells suggests that both CD8αα and CD8αβ dimers are expressed on most T cells [34], or T cells exhibiting lesser responses to equivalent stimulation [75]. This suggests the expression of more CD8α than CD8β on rat macrophages and mast cells [44;53;74] may be a physiological occurence. In sum, while evidence suggests dendritic cells (at least in mouse) do not express CD8β, reasonable evidence supports the contention that rat macrophages, monocytes and mast cells synthesize CD8β and may have CD8αβ heterodimers.

In mouse, CD8 can be expressed as CD8αα homodimers or CD8αβ heterodimers, but not as CD8ββ homodimers [1;76]. However, in human, CD8ββ homodimers have been observed in one [1], but not all studies [27] of cell lines transfected with CD8β. Human CD8ββ homodimers have not been observed on *ex vivo* cells or untransfected cell lines. One group claims to have found CD8ββ homodimers on 40% of human blood γδ TCR T cells [68]. A subpopulation of γδ T cells were bound by anti-CD8β clone 2ST8.5H7, but not an anti-CD8α mAb [68]. Evidence suggests that anti-CD8 mAb clone 2ST8.5H7 binds an epitope depending on co-expression of CD8α and CD8β [77]. Therefore masking of the epitope for the anti-CD8α mAb on CD8αβ may explain the proposed expression of CD8ββ homodimers [68]. The unequivocal discovery of CD8ββ homodimers on normal human cells awaits.

In addition to the complexity of CD8 dimer expression across species and cell types, both CD8α and CD8β may be alternatively spliced. Alternative splicing of CD8α and CD8β also varies across species (Table 1-2). While in mouse alternative splicing of CD8α produces a membrane-bound form lacking most of the cytoplasmic domain (CD8α'), in humans a secreted form of CD8α is produced (CD8α") [78-80]. Mouse CD8α' is found in higher abundance on thymocytes than peripheral T cells [78], whereas in humans CD8α" is secreted at higher levels in activated T cells [79;80]. Up to seven different transcripts of CD8β exist in human with different lengths of cytoplasmic domain; half of them are secreted. These CD8β transcripts are differentially expressed in the thymus and periphery [81;82]. In comparison only two mRNA species are observed for mouse CD8β with three polyadenylation signals (Table 1-2) [76;83;84]. The use of alternatively spliced forms of CD8α and CD8β in different cell types and species is poorly understood, but will be required knowledge to dissect how CD8α and CD8β are adapted and regulated to differentially modulate cell activation and functions.

Several other differences in CD8 among rat, mouse and human species are known (Table 1-2) and will be discussed in more detail in the discussion (Chapter 6).

1.4. Binding to MHC Class I is Fundamental to the Ability of CD8 to Promote Cell Activation

CD8, as a transmembrane, signaling receptor interprets the extracellular environment into intracellular responses. CD8 binding to MHC class I appears to be essential for elicitation of subsequent intracellular signaling. For example, dimunition of CD8 binding severely ablates intracellular phosphorylation of ζ chain and LAT, downstream results of recruitment and activation of CD8-associated lck [24;85-89]. The ability of CD8 to bind MHC class I may be its fundamental requirement to influence cellular responses. Even without the ability to signal through lck or LAT CD8 enhances T cell activation, whether the TCR binds MHC class I or MHC class II [90-92]. Thus, even if CD8 has no signaling function, and binds MHC class I, while TCR recognizes MHC class II, CD8 retains the capacity (albeit reduced) to promote T cell activation. This suggests CD8 may enhance monocyte and macrophage responses by promoting intercellular interactions alone. Indeed, CD8 may bind cellular ligands other than MHC class I and promote cell activation. Carcinoembryonic antigen (CEA), is a highly diverse and glycosylated protein family that can form a complex on the cell surface with CD1d and bind CD8 [93-97]. The region of CD8 that binds CEA and MHC class I may be distinct, and

CD8 binding to CEA depends on glycosylation of CEA [95]. CEA is upregulated in many cancers [97], where it may modulate responses of CTL, macrophages or NK cells to tumor cells by binding CD8.

Given the fundamental ability to bind MHC class I or CEA, CD8 may utilize various mechanisms to activate T cells, NK cells or monocytes. These mechanisms include signaling through lck, LAT, and association with lipid rafts, or receptors like TCR.

1.4.1. CD8α Binds a Conserved Region of MHC Class I Proteins

To analyze the function of CD8 and for other studies in this thesis an overview of MHC class I is required. Tens of proteins with varying degrees of sequence and structural conservation have a tertiary structure derived from or resembling MHC class I. Components of MHC class I structure are used by different proteins in processes from immunity to iron transport and coagulation [98;99]. The MHC class I-fold consists of α1 and α2 domains, formed by two α-helices perched on a β-sheet (Figure 1-3). Endogenous or pathogen derived peptides of 8-15 amino acids are loaded in a groove formed by the two α-helices of the α1α2 domains in the classical immune system MHC class I molecules (Figure 1-3, left). In other proteins with an MHC class I-fold the inter-helical groove may bind lipids [100;101] or may be to narrow to allow binding of any molecule [102]. In most but not all [99;103], MHC class I family members, an immunoglobulin superfamily domain, the α3 domain, sits under the α1 and α2 domains and extends into the transmembrane and cytoplasmic regions (Figure 1-3, right). Finally, β2-microglobulin (β2m) associates non-covalently with MHC class I, excepting some non-classical MHC class I.

The Ig domains of CD8 primarily contact a loop conserved in the $\alpha 3$ domain of MHC class I (Figure 1-3, right; Figure 1-4, left)[2;104]. CD8 binds both classical MHC class I (in human HLA-A, B and C) that exhibit extensive polymorphism within a species, and some non-classical, or more conserved, MHC class I, such as HLA-G, CD1d, and in the mouse TL [94;95;105;106] with an affinity that varies about 2.5 fold [105]. CD8 has a significantly reduced binding affinity for some non-classical MHC class I proteins like HLA-E, and a few classical MHC class I proteins not found in all subjects [105]. Classical MHC class I is expressed on most cell types except red blood cells, while non-classical MHC class I are often restricted in expression to a particular tissue, inflammatory or carcinogenic environment. Thus, classical and non-classical MHC class I ligands for CD8 are ubiquitously expressed. Some non-classical MHC class I proteins like mouse Retinoic acid early inducible protein 1 (Rae1)β and H60 [107] and human endothelial receptor for protein C (EPCR) [99] lack the entire domain containing the CD8-binding site of classical MHC class I. Other non-classical MHC class I molecules like MHC class I polypeptide related sequence (MIC)-A, MIC-B, and Zn-α-2-glycoprotein (ZAG) are not believed to bind CD8 [108;109].

1.4.2. CD8α on Rat Macrophages May Not Bind MHC Class I

The binding of MHC class I to CD8 on monocytes, dendritic cells and NK cells has never been examined directly before the studies in this thesis. Given that CD8 binding to MHC class I may have distinct affinity or avidity on each cell type and stage of activation, these other cell types may offer productive models to discover further variations in CD8 binding to MHC class I. Some studies have suggested CD8 binding to MHC class I is important for CD8 function on NK cells. NK cell effects elicited by cross-linking anti-CD8 mAb could be reproduced with soluble MHC class I [110], or eliminated when MHC class I-/- cells were used [111]. However, other evidence suggests CD8 expressed by macrophages and mast cells may have a novel structure or molecular associations that modulate its binding to MHC class I. When three anti-CD8α mAb were used to detect CD8α on rat macrophages and mast cells a single anti-CD8α mAb (clone G28) did not bind these cells, or bound at significantly lower levels (DG, unpublished)[44;74]. Others have confirmed the relative paucity of binding of anti-CD8α mAb (clone G28) to rat macrophages compared to another anti-CD8α mAb (OX-8) [112]. Clone G28 is immunoglobulin isotype IgG_{2a} which exhibits stronger background binding to FcR at least in human [113]. Thus, despite the use of irrelevant immunoglobulin isotype controls, higher background binding of IgG_{2a} isotype mAb may have obscured the actual binding of anti-CD8α clone G28 to CD8α on macrophages and mast cells. Anti-CD8α clone G28 binds the immunoglobulin-like domain of CD8α that binds MHC class I [33]. This raises the possibility that CD8α on rat macrophages or mast cells may have a novel structure or intermolecular associations that prevents CD8 binding to MHC class I, or changes its affinity or avidity of binding to MHC class I. Thus, it was important to verify the ability of CD8 on rat macrophages and human monocytes to bind MHC class I.

1.4.3. Variations in the Affinity and Avidity of CD8 Binding to MHC Class I May Influence its Function on Monocytes and Other Cells

Whether monocytes express CD8αα or CD8αβ they may have a similar ability to bind MHC class I. Structural and mutational analyses suggest CD8αα and CD8αβ dimers may have slightly different contacts with MHC class I [3]. Nonetheless CD8αα and CD8αβ binding affinity/avidity for MHC class I are similar in many situations, whether they are expressed intact on a cell or in a recombinant form lacking the stalk region [61;114;115].

However the affinity and avidity of CD8αα and CD8αβ binding to MHC class I changes in several circumstances depending on the ligands involved, and the glycosylation of CD8 in a given cell type. Importantly, the affinity/avidity of CD8 binding to MHC class I correlates with the enhancement of T cell activation by CD8 in physiological settings and experimental models [85;86;116-118]. The apparent inability of anti-rat CD8α mAb clone G28 to bind rat macrophages may serve as an indicator of one of these changes that affect CD8 binding to MHC class I. These changes may influence CD8 in its operation according to cell type and inflammatory environment.

1.4.3.1. CD8αα Binds Non-Classical MHC Class I Molecule TL with Higher Affinity than Other MHC Class I

One exception to the postulate that CD8αα and CD8αβ bind identically to MHC class I is CD8αα binding to the mouse non-classical MHC class I molecule TL. TL is expressed on gut epithelial cells, dendritic cells and activated T cells [54]. CD8αα binds TL with 10-fold higher affinity (12 μM) than CD8αβ binds TL or other MHC class I [54;114]. Like CD8αα T cells, the interactions of CD8αα monocytes or dendritic cells with activated T cells and epithelial cells may be enhanced by TL. No human homolog of TL is known [96].

1.4.3.2. Sialylation of CD8 Enhances its Binding to MHC Class I Tetramers

Another exception to the proposal that CD8αα and CD8αβ binds identically to MHC class I involves the lack of sialylation by alpha2,3 sialyltransferase of CD8α [119] and CD8β on immature thymocytes. This lack of sialylation allows CD8αβ to bind MHC class I tetramers with a higher avidity than CD8αα, and without the TCR [60;116;117;120]. Similarly after stimulation of mature T cells, but not T cell clones, CD8 binding to MHC class I tetramers was downregulated in a sialic acid dependent manner [121]. The effect of changes in sialylation of CD8 on MHC class I binding has only been measured with tetramers of MHC class I. Measured changes in MHC class I binding may be avidity effects, related to CD8 clustering for example, rather than a change in CD8-MHC class I binding affinity [122]. Expression of glycosylating enzymes, sialyltransferases among them, is distinctive to each cell type and its activation status. Thus, differential glycosylation of CD8 on monocytes may enable it to interact with MHC class I in ways distinct from CD8 on T cells.

Sialylation of human CD8 on thymocytes appears to parallel that of mouse. In human, immature thymocytes have little alpha2,6 or alpha2,3- sialyltransferase compared to mature thymocytes and this explains the binding of an alpha2,6 sialic acid specific lectin (Maackia amurensis agglutinin) to mature but not immature thymocytes [123;124]. Thus sialylation of human CD8 may also control binding of immature thymocytes to MHC class I as in mouse. In opposition to mouse T cells [121], activation of human T cells may decrease sialylation of CD8 and enhance binding of CD8 to MHC class I. Stimulation of human T cells through CD3 or IL-2 decreases alpha2,6 sialyltransferase activity and robustly induces beta1,6 GlcNAc-transferase [125]. These glycosylation enzymes compete for the same structure, and therefore activation of human T cells may result in decreased sialylation of proteins including CD8 [125], and therefore increased CD8-MHC class I binding avidity [116;117]. Glycosylation of mouse and human CD8 appears to be modified in ways that depend on development and activation state of T cells. Studying the sialylation of CD8 on human monocytes may help elucidate differences in CD8 interactions with MHC class I among cell types that associate with functional differences.

1.4.3.3. Adhesion of CD8 to MHC Class I Is Altered After Cell Activation

In contrast to mature peripheral T cells where TCR activation decreases binding of CD8 to MHC class I in a partly sialic acid-dependent manner [121], TCR activation in T cell clones enhances CD8 adhesion to MHC class I, in a way unrelated to sialylation [126]. Constitutive adhesion of T cells through CD8 to MHC class I attached to a solid surface is undetectable with T cell clones [127]. After activation of T cell clones through the TCR, adhesion of CD8 to MHC class I is detectable – indicating a higher affinity or avidity interaction [4;126]. This "activated adhesion" of CD8 T cell clones can induce CTL cytotoxicity [128], similar to LFA-1 [129]. The affinity and avidity of integrins are enhanced via several means after cell activation [130]. Activated adhesion of CD8 may also depend on affinity and avidity changes [86]. Activated adhesion of CD8 requires phosphatidyl inositol-3 kinase (PI3K) activity and cytoskeletal rearrangement [131;132] suggesting membrane rearrangement or clustering of CD8 results in enhanced avidity of CD8. Movement of CD8 into or out of lipid rafts may regulate CD8 clustering and activated adhesion of CD8, as for integrins [130]. It is possible other receptors that use signaling mechanisms similar to TCR, like FcR on monocytes, may also enhance CD8 adhesion to MHC class I.

1.4.3.4. Activation of Mature T cells Dampens Binding to MHC class I Potentially Through Conformational Changes in CD8

In addition to avidity effects that are sialic acid-dependent and independent, conformational changes in CD8 may change its binding affinity for MHC class I. When T cell binding to MHC class I tetramers was reduced after TCR activation, binding to MHC class I tetramers could not be re-instated to pre-activation levels by removal of sialic acid alone [121]. Anti-CD8α mAb 53-6.7 is believed to induce conformational changes in CD8 and thereby enhance binding to MHC class I tetramers [121;133]. Addition of this anti-CD8α mAb re-instated binding of MHC class I tetramers to pre-activation levels [121]. Thus changes in avidity (e.g. sialylation) and affinity (potential conformational effects reversed by anti-CD8 mAb 53-6.7) may decrease CD8 binding to MHC class I tetramers after activation of mature T cells. Monoclonal antibodies are large proteins with flexible binding sites for antigen. Monoclonal antibodies can cause conformation changes in their ligands, perform enzymatic reactions such as oxygen radical formation [134], or interfere with spatially-distant interactions by steric hindrance. It will be helpful to discover how a select few anti-CD8α mAb can enhance binding of CD8 to MHC class I tetramers [1].

Interestingly, data generated with MHC class I mutated in the CD8 binding site rather than anti-CD8α mAb, also hints that a conformational change in CD8 may regulate binding to MHC class I [86;135]. After activation of T cell clones through TCR, binding of CD8 to MHC class I depended in part on different amino acids in MHC class I [86;135]. Potential conformational changes of CD8 may be transient and dependent on the extra- or intra-cellular environment, making detailed analysis of structure, binding sites and affinity an arduous task. Use of anti-CD8α mAb [133] and mutants of CD8 and MHC class I [86;133;135] to detect and study changes in CD8 conformation in various cell types appears to be the most practical option at present.

The ability of CD8 to activate cell signaling appears to depend on, and be regulated by the affinity and avidity of initial binding to MHC class I [117;118]. Thus, it was important to study factors that may affect binding of MHC class I to CD8 on human monocytes, such as expression of CD8αα versus CD8αβ dimers, sialylation of CD8, and the involvement of CD8 in "activated adhesion". Such studies were performed in this thesis.

1.5. CD8 Interactions with Intracellular Signaling Molecules

CD8 binds to MHC class I, thereby enhancing intercellular adhesion and potentially promoting binding of TCR or other receptors to their ligands. CD8 independent of LAT or lck still promotes T cell activation, presumably by binding MHC class I [92]. Upon binding MHC class I, the effect of CD8 on cell activation may vary depending on whether lck, LAT, or neither is bound to the cytoplasmic domain of CD8. Later, I will discuss at least one means that may regulate whether CD8 binds lck, LAT or neither protein.

When expressed on monocytes, macrophages and dendritic cells CD8 may not signal through both lck and LAT. One obstacle to a wider appreciation of the function of CD8 on macrophages, dendritic cells and monocytes is the presumed lack of lck [136;137] and LAT [138] in these cells. Without cytoplasmic signaling molecules to signal through and no TCR to act as a co-receptor, the mechanism by which CD8 activates myeloid cells is not easily visualized. Thus, I will first analyze evidence that CD8 activates signaling through lck and LAT. Subsequently I will outline evidence suggesting how CD8 can signal in NK cells that have many of the same signaling molecules as T cells, and monocytes, macrophages and dendritic cells, that are thought to lack both lck and LAT. This will take us to the point where we can postulate how CD8 may function on cells other than T cells.

1.5.1. In T cells CD8α Binds Lck

The cytoplasmic domain and transmembrane regions of CD8α and CD8β share the closest conservation among regions of CD8 [139]. CD8 binding to lck has been demonstrated by immunoprecipitation, site-directed mutagenesis, and x-ray crystallography [5;140]. The cytoplasmic domain of CD8α binds lck [141;142] using a zinc-clasp, composed of two cysteines from CD8α, two cysteines from lck and a zinc ion [5]. Because of this CD8 binding to lck can be disrupted by cation chelators and depends on free cysteines.

1.5.2. *CD8α May Associate Directly or Indirectly with LAT*

CD8α co-immunoprecipitates with LAT, and has been proposed to bind LAT [6;60]. However, others have not observed CD8 association with LAT in T cell clones [61]. LAT may bind directly to activated lck [143], and therefore only indirectly to CD8α. Existing data demonstrates that LAT association with CD8 is dependent on the lck binding site in CD8 [6]. If lck binding to CD8α is disrupted, LAT would not co-immunoprecipitate with lck and CD8α. In the presence of excess lck, LAT did not co-immunoprecipitate with CD8 [6]. This could be interpreted as excess lck competing with CD8-associated-lck for LAT. In support of CD8α directly binding LAT, LAT association with CD8α may not be proportional to lck association with CD8α: more LAT co-immunoprecipitates with CD8α than CD4, while the inverse is true of lck [6]. Whether the association of LAT with CD8α is direct will require analysis in lck-deficient cells and with recombinant proteins *in vitro*. At this point effects of CD8 on or through LAT could be interpreted as indirect effects mediated by CD8 binding to lck.

However if CD8 binds directly to LAT, evidence suggests lck and LAT bind the same region of CD8, a membrane-proximal CxC motif, and compete for binding to CD8 [6]. CD8 function would thus be partly regulated through relative availability and competition of lck and LAT for CD8α.

1.6. CD8 Does Not Affect NK Cell Cytotoxicity Alone But May Co-Activate Cytotoxicity with FcR or Other Receptors

Several groups have attempted to activate CD8 on NK cells without co-activation. In these studies the Fc region of anti-CD8α mAb binds FcR on target cells in NK cell killing assays, and excess anti-CD8α mAb is removed. It should be emphasized that in these studies the Fc component of anti-CD8α mAb is masked when it binds FcR on a target cell, and thus is not available to bind FcR on NK cells. As such, CD8α and FcR would not be cross-linked or co-activated. In these experiments no cytotoxicity is induced through CD8 ligation alone, whereas cytotoxicity is induced by ligation of CD16 or CD69 [111;144;145]. CD8 function on T cells, at least for the induction of CTL-mediated cytotoxicity, requires co-activation through a second receptor, the TCR. Because of this, CD8 may not signal alone on NK cells. CD8 may require co-activation of NK cells through another receptor (Figure 1-5, Table 1-3).

As NK cells and T cells express many of the same signaling proteins, CD8 may co-activate NK cell receptors analogously to its co-activation of TCR responses on T cells. However, CD8 probably does not co-activate all receptors that activate NK cell killing as anti-CD8α mAb does not inhibit NK cell cytotoxicity when used in a way that inhibits CTL cytotoxicity [35]. NK cell receptors may contain an ITAM in their cytoplasmic domain or be attached to signaling mechanisms including DAP10/12, CD3ζ or common γ chain. Analogously to CD8 co-activation of TCR responses in T cells, CD8 may selectively co-activate NK cell receptors that use lck or LAT and CD3ζ. CD16, CD32, two low affinity FcγRs and FcμR for IgM [146], expressed by many NK cells signal through Syk [146], ZAP-70 [146], CD3ζ [147], lck [148-150], and probably LAT [151], and thus use most of the signaling components associated with CD8-dependent signaling in T cells (Table1-3, Figure 1-5). In fact, CD8 on NK cells co-immunoprecipitates lck and CD3ζ [141]. Consequently it is plausible that CD8 could amplify NK cell activation through several FcR (Figure 1-5).

1.7. On Myeloid Cells CD8α May Signal Via Common γ chain, Syk and LAT

NK cells are known to express lck, LAT, and CD3ζ and thus are, in theory, capable of signaling through CD8 in the same way as T cells, after co-activation through a second receptor (Figure 1-5). A major unanswered question is what proteins CD8 binds intracellularly in CD8+ monocytes, macrophages and dendritic cells, as these cells are presumed to not express lck [137;152], LAT [153] or CD3ζ. However, some data suggests lck and LAT may be expressed by monocytes and other myeloid cells (see below), suggesting CD8 may signal through the same proteins in both monocytes and T cells. Alternatively, other data suggests that if lck is not expressed in monocytes, macrophages and dendritic cells, CD8 may signal via LAT alone.

CD8α binds lck [5]. Lck phosphorylates CD3ζ recruiting ZAP-70 which then can phosphorylate LAT. CD3ζ is thus an important part of CD8 mediated signaling through lck and LAT. CD3ζ expression is thought to be restricted to T cells and NK cells. However, CD3ζ is part of a family of proteins generated by ancestral duplication, that includes common γ chain [154]. CD3ζ and common γ chain conserve their exon-intron organization [154;155] and have signaling regions exhibiting 44% homology [156]. Common γ chain can substitute for CD3ξ to promote the expression of TCR on immature T cells and CD8α+ intestinal T cell subpopulations [157;158]. CD3ζ and common γ chain both function with FcR [159] [160], NKp30, and NKp46 [161] in NK cells, FcεRI in mast cells [162] and the TCR in some γδ T cells [163] (Table 1-3). Most importantly, common γ chain can substitute for CD3ξ in T cell activation through TCR [164], suggesting, as shown elsewhere [151], that common γ chain operates adequately with TCR signaling pathways like lck and LAT. In fact in some mature effector T cells Syk and common γ chain replace ZAP-70 and CD3ζ in TCR signaling [165]. CD8 on monocytes, NK cells, and dendritic cells may signal in conjunction with common γ chain associated receptors as well as CD3ζ associated receptors (Figure 1-6).

ZAP-70 associates with CD3ζ and is an important upstream component of TCR-CD8 signaling. While ZAP-70 is restricted to T cells and NK cells, the ZAP-70 homolog Syk is found in monocytes, dendritic cells, mast cells, NK cells and some T cells. ZAP-70 can be more or less functionally replaced by Syk [166;167], suggesting Syk and ZAP-70 are similarly activated by upstream signaling through TCR and potentially CD8 or CD4, and will similarly propagate responses. Evidence suggests signaling pathways that CD8 activates in macrophages depend on Syk kinase. For example, anti-CD8α mAb induced TNF release from rat macrophages was attenuated by inhibition of Syk kinase using anti-sense RNA or piceatannol [44;53]. Thus, CD8 signaling may be Syk-dependent in cells that do not express ZAP-70.

The src kinase lck is considered a lymphocyte specific protein [136;137]. However others have found that a variety of monocyte/macrophage-(like) cells express lck, including the human monocytic cell line THP-1 [168], dendritic cells (Shannon Turley, personal communication), Schwann cells [169] and microglia [170]. In much the same way LAT is thought to be expressed in T cells, NK cells, and interestingly in CD8+ mast cells, but not in other myeloid cells [138]. However others found that the monocyte cell line THP-1 uses LAT to signal through FcγR chain, and LAT-deficient mouse macrophages had diminished FcγR-mediated responses [151]. Thus, LAT and lck expression in CD8+ monocytes, macrophages, or dendritic cells warrants re-examination. LAT and lck may be expressed in monocytes and macrophages at reduced levels or only when these cells are differentiated under certain conditions, perhaps at particular locations in the body.

If the signaling molecules that CD8 functions through (CD3 chains, lck, LAT, ZAP-70) in T cells are not found, even at low levels, in monocytes and macrophages, how might CD8 induce cell activation? Rat macrophage release of TNF and IL-1β was attenuated with the inhibitor of src family tyrosine kinases PP1. However, this does not define which src kinase is involved in rat macrophage responses mediated by CD8. CD4 and CD8 bind through a similar mechanism to lck [5]. Others found that lck kinase activity did not precipitate with CD4 from rat macrophages [141], suggesting CD8 on rat macrophages does not bind lck. Sequence analysis gives no insight into what other src kinase could bind CD8 in macrophages, monocytes or dendritic cells. Src kinases are highly divergent in the N-terminal region, and the motif in lck that binds CD8 is not conserved in another known src kinase. CD4 on monocytes was recently shown to co-immunoprecipitate with src kinase hck [171]. Hck is an important signaling component of monocyte activation through high and low affinity FcγR [172-175], signaling pathways that may involve CD8 on monocytes according to this thesis. The binding of lck to CD8 depends on the unique N-terminus of lck [5] that does not appear to have homology to hck. As a result, it is likely that if CD8 binds hck it is indirectly, or through a mechanism dissimilar from lck.

CD8 may bind LAT [6], but the region of LAT that interacts with CD8 has not been characterized. It is possible that CD8 could bind other adaptor molecules, such as NTAL in addition to LAT in myeloid cells. In an interesting study, three proteins of 35, 45 and 55 kDa co-immunoprecipitated with CD4 from a human monocyte cell line (THP-1) [176]. The 35 and 55 kDa proteins may be LAT (36-40 kDa) and lck (or hck) (56-60 kDa) as these proteins have been described in THP-1 and bind CD4 [151;168].

If CD8 co-signals with receptors other than TCR in monocytes, macrophages and dendritic cells, it may do so using LAT alone rather than lck and LAT. When expressed in T cells, Syk, unlike ZAP-70 does not require lck to become activated after TCR stimulation [167]. Thus if CD8 co-activates Syk-dependent receptors in monocytes, lck may not be required. What is more, Syk can phosphorylate and activate LAT [177;178], suggesting CD8 could participate in responses after initial priming of a Syk-dependent receptor. LAT is required for signaling through several common γ chain linked receptors such as FcγR [151;179], FcεRI on mast cells [180], ILT1 (and potentially other ILT family receptors) [151;181], and gpVI [182-184] (Table 1-3). The activating receptor for MHC class I, PIR-A requires the common γ chain for its intracellular signaling, suggesting it may also signal through Syk and LAT [185] (Table 1-3). Thus CD8 may be able to function with Syk/common γ chain-dependent receptors as with ZAP-70/CD3ξ-dependent receptors, like TCR (Figure 1-6). CD8 may partner with several receptors to co-activate cells (Table 1-3) including FcR, ILT1, PIR-A, NKp30, and NKp46 (Table 1-3, Figure 1-6). As a model system to test some of these postulates, studies were performed in this thesis to examine the ability of CD8 to co-activate monocyte responses through FcR and LAT.

It is possible CD8 co-signaling function may extend to DAP10/12 associated receptors that use Syk such as SIRPβ [186] and integrins. In one intriguing isoform of human CD8β mRNA, two transmembrane regions are predicted (accession: NP_742097.1) [187]. The second transmembrane region contains two lysine residues that in the context of other receptors interact with immune-activating adaptor molecules like DAP10/12 [188] and common γ chain. If this CD8β mRNA is translated CD8αβ may have the ability to activate cells independently by linking LAT/lck to DAP10/12 or common γ chain and Syk. Alternatively, human CD8ββ dimers containing this two transmembrane isoform of CD8β, expressed on an unidentified cell type, may instigate responses through DAP10/12 or common γ chain and Syk.

1.8. Summary and Aims of Thesis Research

In this brief section I will summarize the evidence and rationale that determined the research performed in this thesis.

Evidence suggested CD8 expressed by rat macrophages may significantly contribute to pathology of several diseases through production of pro-inflammatory mediators. However, as mouse monocytes and macrophages appeared to be CD8α -ve the relevance of observations made in rats to human health was uncertain. Thus, the first aim of this thesis was to determine whether CD8 was expressed by human monocytes or macrophages. Human monocytes were chosen as a model to examine CD8 expression.

The second aim of this thesis was to uncover how CD8-mediated release of pro-inflammatory mediators by monocytes might be regulated. Given that TCR and FcγR share analogous and overlapping signaling mechanisms and that CD8+ rat monocytes were found in significant numbers in several diseases whose severity depended on FcγR a model of how CD8 might co-activate FcγR responses was developed. The ability of CD8 ligation to enhance FcγR responses of monocytes was tested.

A third aim of this thesis was to identify the intracellular molecules CD8 signals via in monocytes. Notably, monocytes are not widely appreciated to express lck or LAT which are the only known intracellular signaling partners of CD8. Literature suggested LAT was the most likely candidate for CD8-mediated signaling in monocytes; therefore phosphorylation of LAT was measured after activation of CD8-dependent responses in monocytes.

As a fourth aim of this thesis, several approaches were taken to discover potential novel forms of CD8 protein on monocytes. Some literature hinted that CD8 on monocytes, macrophages and T cells may differ, however whether these differences in CD8 were due to post-translational modification, alternative splicing, inter-molecular associations, or even conformational changes was uncertain. 2-D electrophoresis of CD8 on monocytes and T cells was performed. Sialylation of CD8, which is known to influence CD8 function and T cell activation, was studied on monocytes. Much work was devoted to purifying and identifying by mass spectrometry proteins recognized by anti-CD8α mAb that had a higher Mr than expected for CD8α.

The ability of CD8 to bind MHC class I is regulated by several means in several situations and some evidence suggested that the MHC class I binding region of a potentially novel form of CD8α on rat macrophages might be inaccessible and thus not bind MHC class I. Thus as the fifth aim, the ability of CD8 on rat macrophages and human monocytes to bind MHC class I was tested.

A sixth aim of this thesis evolved from experiments performed for aim five (above) that suggested the cytokine macrophage migration inhibitory factor (MIF) may have some structural similarity to MHC class I. My attention was piqued by reports that demonstrated MHC class I-like functions for MIF, such as inhibition of NK cell killing. The sixth aim was thus to determine the extent and structural location of similarity between MIF and MHC class I and subsequently to determine if this similarity could account for MHC class I-like functions of MIF.

1.9. Tables

	αβ T cell	δγ T cell	NK cell	Monocyte	Macrophage	Mast cell	Dendritic cell
Human	+++	+++	+ [34-37]	?(P) [51;52]	? (P) [48-50]	+ [189]	+++ [31]
Mouse	+++	+++	- [36]	- [47]	? (P) [30]	?	+++ [30]
Rat	+++	+++	+ [32;33]	?(P) [51;190]	+ [44]	+ [74]	+++ [28;29]

Table 1-1, Part I. Expression of CD8α among cell types and species. Expression of CD8 is denoted by a + symbol where evidence of synthesis of CD8 by the cell type in the given species is available. Number of + symbols denotes relative level of protein on cell surface. (P) denotes situations where protein has been shown on the cell surface, but there is no evidence that the protein is synthesized by the cell. NK cell and monocyte expression of CD8α is highlighted to emphasize the apparent differences among species in the expression of CD8α on these cells.

	αβ T cell	δγ T cell	NK cell	Monocyte	Macrophage	Mast cell	Dendritic cell
Human	+++	?(P) [68]	- [34]	?	?	+ [189]	?
Mouse	+++	+++ [67]	-	-	? [30]	?	- [30]
Rat	+++	+++ [66]	+ [33]	?(P) [51]	+ [44]	+ [74]	?

Table 1-1, Part II. Expression of CD8β among cell types and species. Although CD8ββ homodimers may be expressed in humans (Devine et al. *J. Immunol.* 164: 833), this has not been described in untransfected cells: therefore listing of CD8β implies expression of CD8αβ heterodimers. Expression of CD8 is denoted by a + symbol where evidence of synthesis of CD8 by the cell type in the given species is available. Number of + symbols denotes relative level of protein on cell surface. (P) denotes situations where protein has been shown on the cell surface, but there is no evidence that the protein is synthesized by the cell. The respective CD8 chain is expressed on a subpopulation of cells except macrophages where a homogenous population of cells appears to express low levels of CD8β.

	TL High Affinity Ligand for CD8αα	Syk Expressed in T cells	*Alu* Repeats in CD8α Enhancer, Promoter	DNA Hypersensitivity Sites Controlling CD8α Expression	CD8α Splicing	CD8β Splicing	CD8ββ Dimers	CD8α Exons
Human	-	+ [191;192]	+ [193]	[36;193;194]	Secreted [79;80]	5-7 [81;82]	+ [1]	6
Mouse	+ [54]	- [191;192]	-	[194]	Membrane bound [78]	2 [83;84]	- [1]	5
Rat	-		-		?	?		

Table 1-2. Other known differences among species in CD8 and CD8-associated signaling mechanisms and ligands.

	Signaling through Lck	Signaling through LAT	Signaling through γ Or ζ chain	Association with CD8 on the Cell Surface	Induced Fit Binding/ Slow On-Rate	Potential TCR Ancestry
TCR	+	+	+	+	+	+
CD16	+ [148;150]	+ [151;179;195]	+[146;147;160]			
CD32	+ [149]		+ [146]			
CD64		+ [151;195]	+			
FcμM			+[146]			
ILT1		+	+ [181]			
FcεR1		+ [180]	+[162]			
Nkp30/46			+ [161]			
Ly49					+ [196]	
MHC Class I			Signal through Syk [197-199]	+		
CD81				+		
CD36				?		
CRTAM				?		+ [200]
SIRPβ		+				+ [200]

Table 1-3. CD8 may co-activate responses of several receptors besides TCR. CD8 may enhance cell activation by signaling with several receptors. If a receptor is noted to signal through γ or ζ chain, it assumedly also uses Syk or ZAP-70, and should have the potential to signal through lck or LAT, provided these latter two are available in the cell of interest. CD8 may promote binding of some receptors that have a slow on-rate to their ligands.

1.10. Figures

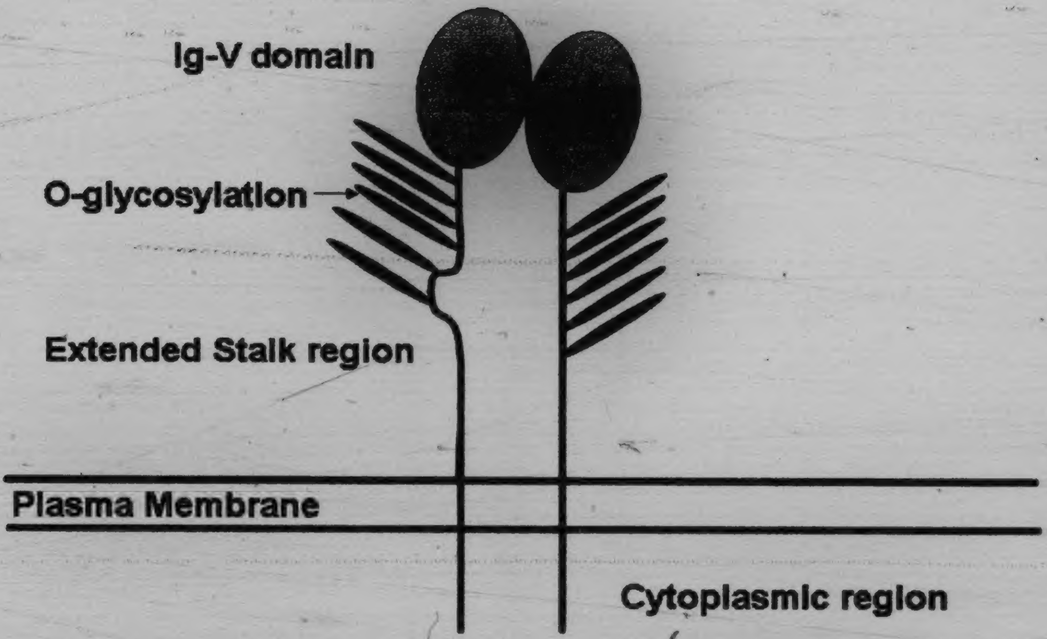
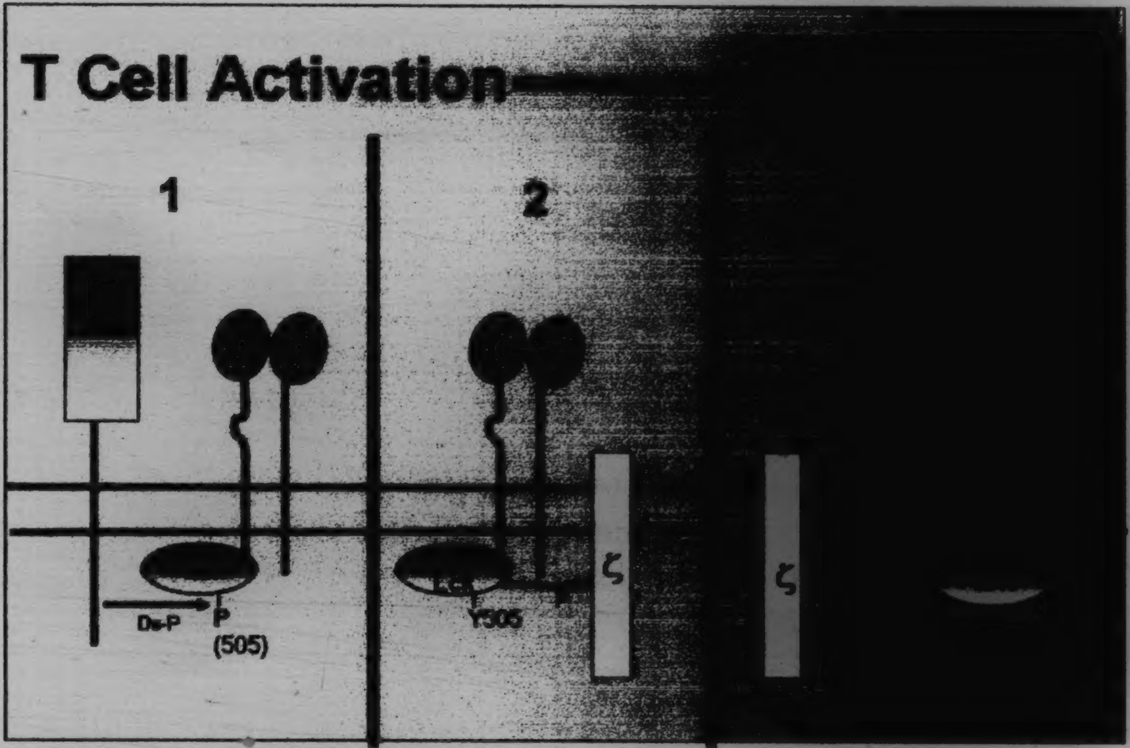

Ig-V domain
O-glycosylation
Extended Stalk region
Plasma Membrane
Cytoplasmic region

Figure 1-1. Overview of CD8 structure. CD8α or CD8β may be expressed as CD8αα or CD8αβ dimers. The general structure and glycosylation of CD8α and CD8β are similar. Amount and type of O-glycosylation is expected to vary among cell types.



T Cell Activation
1
2
De-P
P
(505)
Y505
ζ
ζ

Figure 1-2. CD8α bound to lck and CD8α associated with LAT are involved at distinct temporal stages of T cell activation.

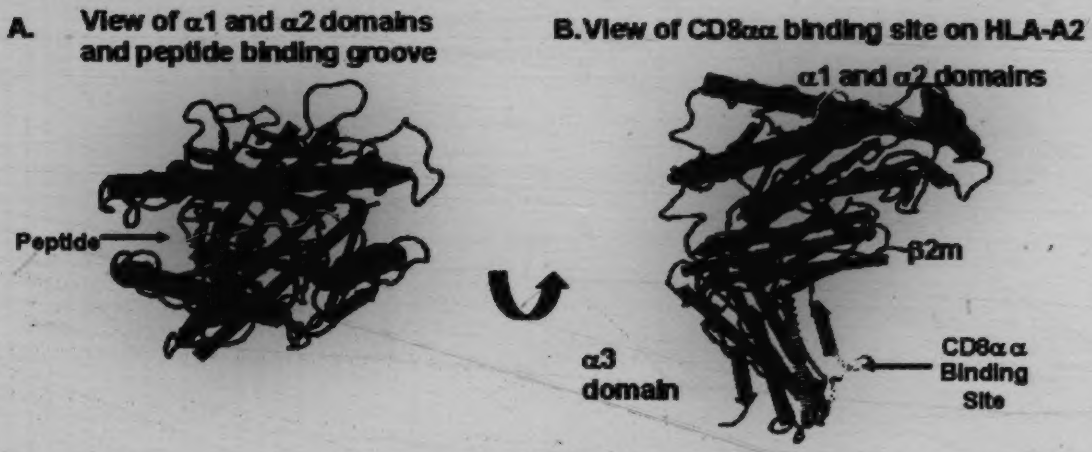

A. View of α1 and α2 domains and peptide binding groove
Peptide
B. View of CD8αα binding site on HLA-A2
α1 and α2 domains
β2m
α3 domain
CD8αα Binding Site

Figure 1-3. Representative structure of MHC class I showing peptide binding groove and CD8αα binding site. (A) Peptide (highlighted in yellow) binds in groove formed by α helices from α1 and α2 domains, sitting on top of a β sheet. Each TCR may contact slightly varied parts of α helices and peptide. (B) Main CD8α binding site in the α3 domain of MHC class I is highlighted in yellow. Peptide remains highlighted in yellow as in (A) to provide orientation. Shown is crystal structure of human MHC class I HLA-A2 bound to CD8αα (PDB: IAKJ) and TCR bound to HLA-A2 (PBD: 1BD2)

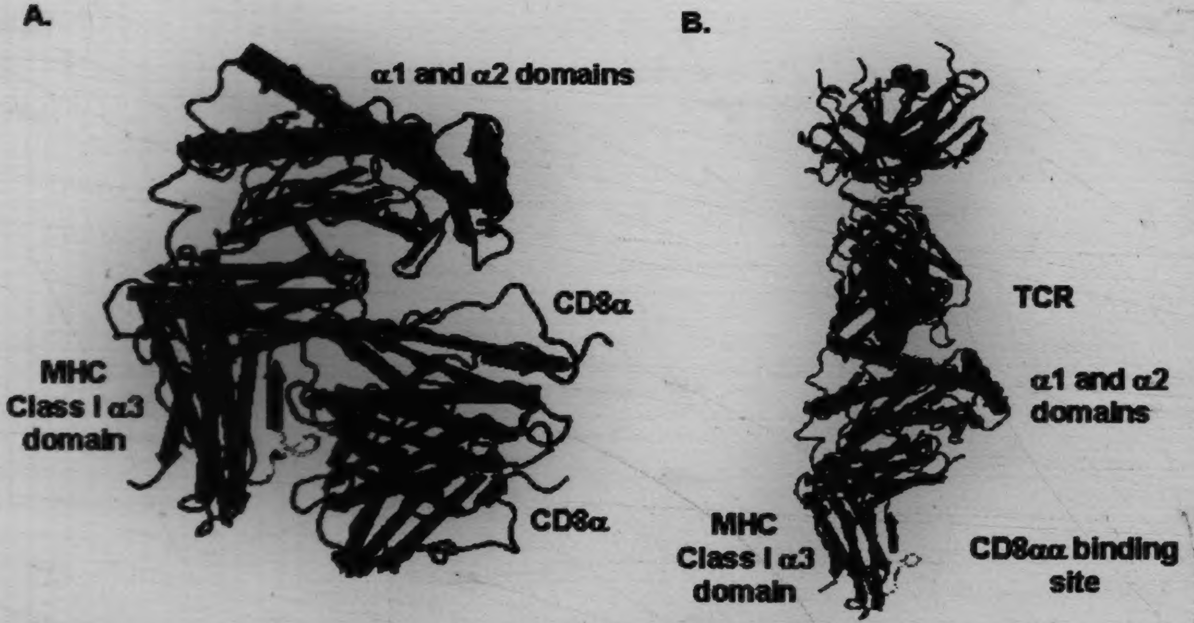

A.
α1 and α2 domains
CD8α
MHC
Class I α3
domain
CD8α
B.
TCR
α1 and α2
domains
MHC
Class I α3
domain
CD8αα binding
site

Figure 1-4. Representative structure of MHC class I showing binding sites and orientation of CD8αα and TCR. Notice the different orientations TCR and CD8αα adopt to bind MHC class I. (A) A CD8αα dimer bound to MHC class I (PBD: 1AKJ). Main CD8αα contact residues on MHC class I are highlighted in yellow as in Figure 2. (B) TCR bound to MHC class I. Peptide and CD8αα binding site are highlighted in yellow.

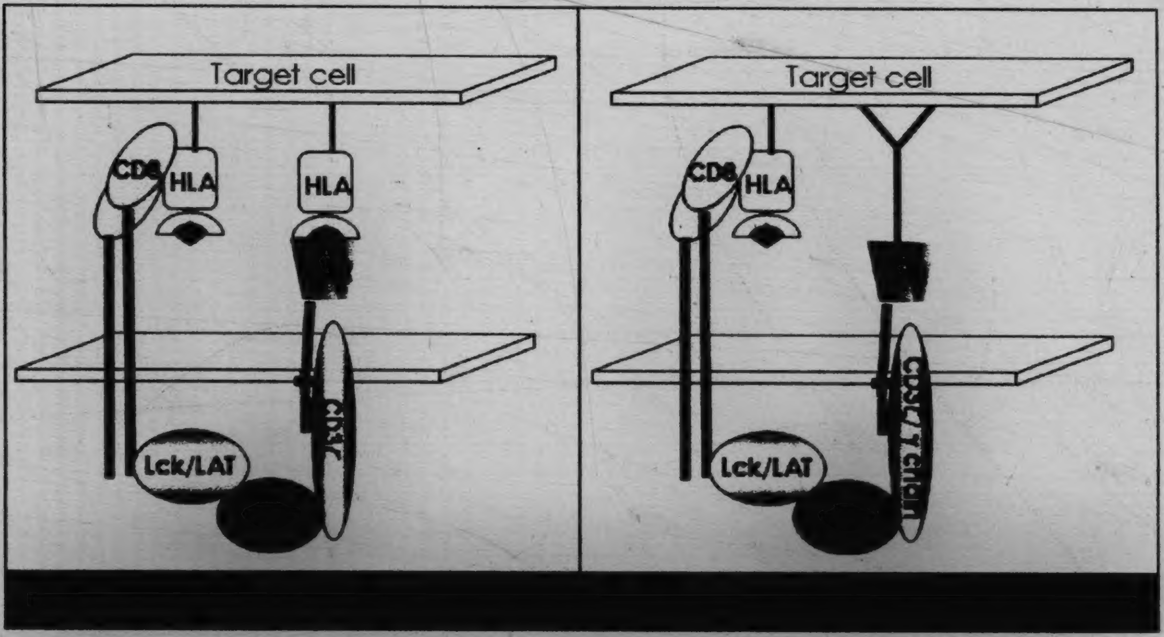

Target cell
CD8
HLA
HLA
CD3ζ
Lck/LAT
Target cell
CD8
HLA
CD3ζ/γ chain
Lck/LAT

Figure 1-5. NK cell activation through FcR uses the same signaling mechanisms that CD8α complements and co-activates in T cells. CD8 recruits lck or LAT to proximal TCR signaling involving CD3ζ and ZAP-70. In NK cells, the proteins that CD8-complements and co-activates in TCR signaling, CD3ζ and ZAP-70, are used in FcR signalling. CD8 may co-activate NK cell signaling through FcR.

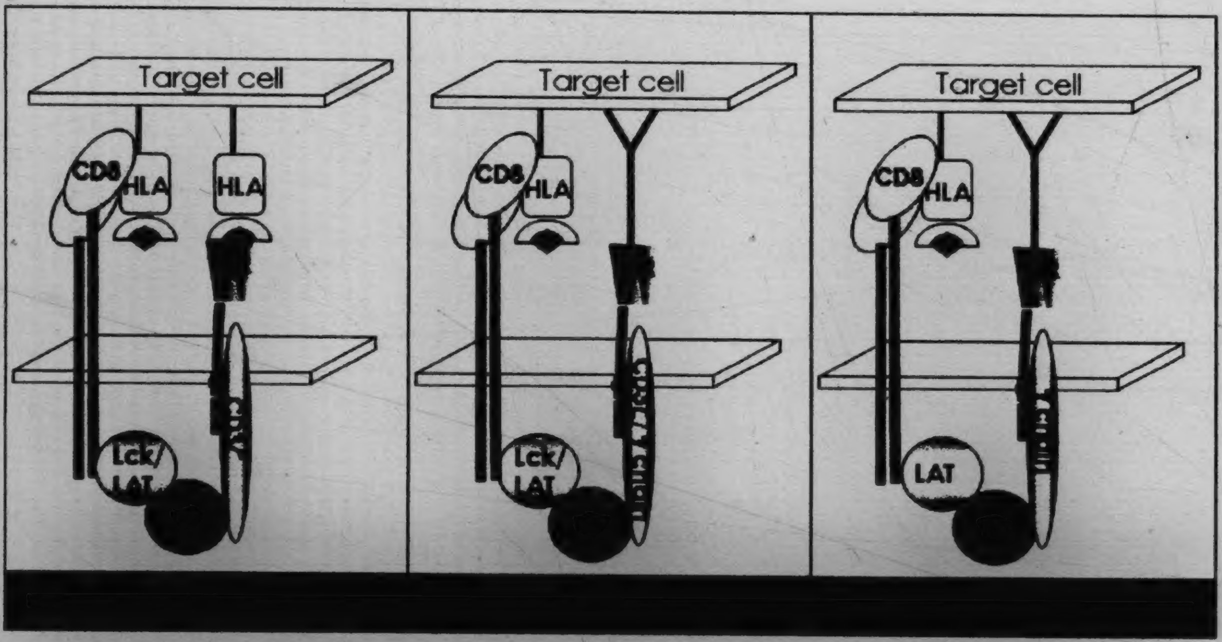

Target cell
CD8
HLA
HLA
Lck/
LAT
Target cell
CD8
HLA
Lck/
LAT
Target cell
CD8
HLA
LAT

Figure 1-6. CD8$\alpha\alpha$ on monocytes and dendritic cells may co-activate FcR-initiated responses. Evidence discussed in the text suggests CD8 associated with LAT, may participate in signaling through FcR in monocytes, macrophages and dendritic cells. Lck may not be expressed in monocytes, but evidence suggests replacement of ZAP-70 with Syk may obviate the need for lck.

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2. LIGATION OF CD8α EXPRESSED BY HUMAN MONOCYTES ENHANCES FCγR-DEPENDENT TNF RELEASE AND LAT PHOSPHORYLATION

Running Title: CD8α Expressed by Human Monocytes Enhances FcγR Responses

Derrick J. Gibbings, Marcelo Marcet-Palacios, Yokananth Sekar, Eric Wong and A. Dean Befus

Pulmonary Research Group, Division of Pulmonary Medicine, Department of Medicine, University of Alberta, Canada

Corresponding author: A. Dean Befus
550 Heritage Medical Research Centre
University of Alberta
Edmonton, Alberta
Canada T6G 2S2
Telephone: 780 492 1909
Fax: 780 492 5329
dean.befus@ualberta.ca

Supported by Canadian Institutes of Health Research (CIHR) and Alberta Lung Association operating grants (ADB), an Alberta Lung Association Studentship award to DG and summer studentship award (MN) from the Alberta Heritage Foundation for Medical Research

Abbreviations: AM (alveolar macrophage), Mϕ (macrophage), immunoglobulin-like-transcript (ILT), peripheral blood mononuclear cell (PBMC)

Keywords: Human, Monocytes/Macrophages, CD8, MHC Class I, TNF, FcγR, LAT, Linker for Activation of T cells, tetramers

2.1. Introduction

CD8α is a surface glycoprotein typically associated with a subpopulation of cytotoxic T cells (CTL) [189]. An alternatively spliced form of human CD8α (secreted CD8α, sCD8α) without the transmembrane region can be secreted from T cells [190;191]. On T cells CD8α occurs as a homo- or hetero-dimer, αα or αβ [192], and participates in CTL responses. The cytoplasmic domain of CD8α binds the src kinase lck [193] and potentially the adaptor protein Linker for Activation of T cells (LAT) [194] to enhance CTL activation through TCR.

CD8 enhances T cell responses 10-100 fold when it activates T cells with a MHC class I binding TCR, but only 2-9 fold when it activates T cells with a MHC class II binding TCR [195-197]. This suggests that even without classical co-receptor activity wherein CD8α may bind the same MHC class I as TCR, CD8 can enhance responses 2-9 fold by participating in activation of intracellular signaling and other adhesion affects. CD8 is recruited to the site of TCR activation even if it cannot bind MHC class I [198;199], further emphasizing that CD8 does not need to bind the same ligand as TCR to participate in cellular responses.

CD8 on T cells co-activates responses initiated by TCR, but no such co-activating role has been described for CD8 on other CD8+ cells like dendritic cells [200], NK cells [192;201], mast cells [202] or macrophages [203]. Interestingly, the Fcγ chain, a component of several FcR can substitute for CD3ξ in T cell activation through TCR, suggesting it operates adequately with TCR and CD8 signaling pathways involving lck and LAT. In fact in some mature effector T cells, Syk and Fcγ replace ZAP-70 and CD3ζ in TCR signaling [204]. Accordingly, CD8 may co-activate responses through γ chain-linked receptors like FcR.

The cell types that express CD8α differ among mice, rats and humans. While human [201] and rat NK cells express CD8α, mouse NK cells do not [205]. Rat Mφ and monocytes express CD8α and CD8β [203;206], however, our efforts and those of others to detect CD8α protein on mouse monocytes and Mφ have been unsuccessful [207;208]. Two recent studies identified binding of anti-CD8α mAb at high levels to a small percentage of human monocytes during immune responses [206;209]. Neither study queried whether lower levels of CD8α were found on these cells, only one study used isotype mAb to control for binding of anti-CD8α mAb, and neither study demonstrated that monocytes synthesize CD8α, or that CD8α on these cells was functional. A portion of CD8α and all the CD8β found on mouse dendritic cells is derived from T cells [210]. As such, CD8α observed on human monocytes in the two studies cited above may be acquired as soluble CD8α released from CD8+ T cells [211], or with membrane from CD8+ T cells [212], in which case CD8α may be incapable of inducing intracellular signaling and monocyte activation.

CD8+ cells are determinative components of some diseases, as for example, depletion of CD8α+ cells with mAb in rat models ameliorates spondyloarthritis [213] and glomerulonephritis [214]. CD8α+ monocytes and macrophages (Mφ) have been observed in these rat models [213;215], and TNF is an important mediator of the pathology [216-218]. We established that cross-linking CD8α on rat Mφ using mAb induced TNF, IL-1β and nitric oxide production and markedly activated Mφ for anti-microbial activity [219;220]. CD8+ monocytes in rat have enhanced cytotoxic functions compared to CD8- monocytes [221;222], although the role of CD8 in this cytotoxicity was not examined. If CD8 is expressed on monocytes and Mφ, these cells may account, at least in part, for functions previously attributed to CD8+ T cells.

In this report, we establish using several methods and six mAb that human monocytes express CD8α. We provide evidence that CD8α on monocytes mediates binding to MHC class I and co-operatively promotes monocyte activation, TNF release and LAT phosphorylation through FcR dependent mechanisms.

2.2. Materials, and Methods

2.2.1. Antibodies

Isotype control antibodies were mouse IgG_1 and IgG_{2a} (Sigma, St. Louis, MO), IgG_{2a}-FITC, and -PE, (Caltag, Burlingame, CA). Anti-CD8α mAb used were: D9 and 32-M4 (Santa Cruz, Santa Cruz, CA) LT8 (Serotec, Raleigh, NC), B9.11 (Beckman-Coulter Canada Inc., Mississauga, ON), and Nu-Ts/c (Nicherei Corp., Tokyo, Japan). Polyclonal anti-human CD8α Ab (H160) was obtained from Santa Cruz. Anti-CD8α mAb 51.1 (gift of Dr. D. Burshtyn, University of Alberta) and OKT8, and anti-rat MHC class I mAb OX18 (European Collection of Cell Cultures, Salisbury, UK) were purified from hybridoma supernatant by protein G affinity chromatography. Anti-CD8β- antibodies were obtained from Beckman-Immunotech (clone 2ST8.5H7-PE, Mississauga, Canada) and Serotec (clone 5F2). Anti-CD3-FITC and anti-CD14-FITC/PE were obtained from Caltag. Anti-mouse Ig-FITC (STAR70) was obtained from Serotec. Anti-CD69 mAb and matching isotype control were obtained from BD Biosciences. Anti-mouse Ig-HRP was purchased from Pierce (Rockford, IL).

2.2.2. Cell recovery and culture

The promonocytic cell line THP-1 [223] was maintained in American Type Culture Collection recommended media (RPMI 1640 medium, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate 0.05 mM 2-mercaptoethanol, and 10% fetal bovine serum [FBS]). CTL clones [68] were a gift of Dr. Chris Bleackley (University of Alberta).

Human blood (100 mL) was collected into heparanized tubes. Red blood cells were sedimented by addition of 7 mL 6% dextran (Sigma) in RPMI 1640 per 35 mL blood (0.5 h, room temperature). White blood cells and plasma were underlayed with Ficoll-Paque Plus (Amersham Biosciences, Oakville, ON, Canada) and centrifuged (room temperature, 25 min, 200 g) without brake. The interface layer was removed and washed three times in PBS to obtain blood mononuclear cells (PBMC). Monocytes were enriched by three methods. Greater than 80% enriched monocytes were obtained from a Percoll gradient [224] for studies of monocyte activation. Monocytes enriched by Percoll were further purified (>99%) by anti-CD14-PE flow sorting for western blot and RT-PCR analysis. Macrophages were differentiated from adherent monocytes (1 h, 37 C) with 500 ng/mL GM-CSF (Biosource, Camarillo, CA) for 3 d.

Human bronchoalveolar lavage (BAL) cells were collected at the University of Alberta Hospital by Dr. Wong with informed consent of patients. Ethics approval was granted by the University of Alberta/Capital Health Research Ethics Board. BAL samples were from patients with preliminary diagnoses of sarcoidosis (2), pulmonary nodules, bilateral infiltrates and lingula squamous cancer.

2.2.3. *Flow cytometry*

Cells were kept on ice throughout experiments. Non-specific mAb binding to cells was minimized with 5% milk, 0.1% bovine serum albumin (BSA) in PBS. In some experiments human Ig (50 μg/mL, Bethyl Laboratories Inc., Montgomery, TX) was used to minimize binding of mAb to FcR. Cells were treated with 10 μg/mL isotype mAb or anti-CD8α mAb, washed three times and incubated with anti-mouse Ig-FITC Ab (1/100, STAR70, Serotec).

Anti-CD8β mAb 2ST8.5H7 directly conjugated to PE was used at 10 μg/mL and compared to IgG2a-PE. Cells were washed three times and incubated with 1/10 normal mouse serum and before addition of anti-CD14-FITC (1/50).

To analyse the contribution of high affinity FcγRI to anti-CD8α mAb binding to monocytes, PBMC were pre-incubated for 30 min with anti-CD64 mAb clone 10.1 (10 μg/mL, BioLegend, San Diego, CA), which blocks binding of Ig to CD64 [225]. Cells were washed and incubated with IgG2a-PE (10 μg/mL, BD Biosciences) or 32-M4-PE (10 μg/mL, Santa Cruz). Cells were washed and data was collected on a FACScan.

All flow cytometry analysis was performed with WinMDI and CellQuest Pro (BD Biosciences) programs. Monocytes were gated by characteristic FSC/SSC scatter and high expression of CD14. AM were selected by large FSC/SSC values and autofluorescence in FL2 channel as described [69].

2.2.4. *Confocal Microscopy*

PBMC were adhered to poly-L-lysine coated coverslips for 0.5 h, fixed with 4% paraformaldehyde (10 min) and permeabilized with 0.1% triton-X-100 in PBS (10 min). Cells were blocked (10% FBS, 3% BSA, 30 min) before staining with anti-CD8α mAb (10 μg/mL). Cells were washed three times (5 min, 2 mL PBS) between each reagent. Cells were sequentially incubated with anti-mouse-Ig-rhodamine red (Molecular Probes, Eugene, OR), 1/10 normal mouse serum and anti-CD14-FITC or anti-CD3-FITC (Caltag). Images were obtained using an Olympus FV1000 confocal microscope (Carsen Group, Markham, ON) with Fluoview software.

2.2.5. *RT-PCR*

Total RNA was isolated using the Qiagen RNeasy kit. RNA was reverse-transcribed with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) using oligo(dT) as primers. Thereafter, PCR was performed in 20 μl reactions with primer pairs (25 μM) below. Intron-spanning primers of the sequence 5'-TTTCGGCGAGATACGTCTAACCCTGTGC-3' and 5'-TTTAGCCTCCCCCTTTGTAAAACGGGCG-3' were used to generate a CD8α cDNA fragment of 379 bp [70]. Intron-spanning primers generating a 209 bp product for CD8β were 5'-GGTGAAGAGGTGGAACAGGA-3' and 5'-CTTGAGGGTGGACTTCTTGG-3'. Intron-spanning primers for LAT generating a 532 bp product were 5'-GTATCCAAGGGGCATCCAGTT-3' and 5'-CCTCTTCCTCCACTTCCTCTG-3'. A β-actin cDNA fragment of 326 bp was produced using intron-spanning primers of sequence 5'-GGC ATC CTC ACC CTG AAG TA-3' and 5'-AGG GCA TAC CCC TCG TAG AT-3'. PCR amplification was performed for 35 cycles of 1 min at 94 C, 1 min at 60 C and 2 min at 72 C, and a final cycle of 72 C for 10 min to complete polymerization. PCR products were run on a 1.5% agarose gel containing ethidium bromide. Intron-spanning primers were used and samples were treated with 84 U/μL DNase I before RT-PCR to avoid interference of contaminating DNA in purified RNA.

Samples used to amplify CD8α mRNA were also amplified with intron-spanning CD3ζ RT-PCR primers (5' – GCACAGTTGCCGATTACAGA – 3' and 5' – GCCACGTCTCTTGTCCAAA – 3', 293 base pair product) for 50 cycles, performed as above.

2.2.6. 2-D electrophoresis

Lymphocytes and monocytes were enriched by collecting non-adherent and adherent cells after 1 h in culture flasks. Lymphocyte and monocyte lysates were prepared using the 2-D cleanup kit (Bio-Rad) and resuspended in IPG strip rehydration buffer (Bio-Rad) with 2% carrier pI 3-10 ampholytes (Bio-Rad) for analysis. Lysates were focused on 7 cm pI 3-10 strips (Bio-Rad), at voltage maximums of 50 V 10 min, 250 V 30 min, 750 V 1 h, and 8 000 V for 13 000 V h. Strips were equilibrated, reduced, and treated with iodoacetamide according to supplier directions (Bio-Rad) before running second dimension.

2.2.7. Monoclonal antibody affinity chromatography for purification of rat MHC class I

OX18 (anti-rat MHC class I) at 5-10 mg/mL in 0.1 M HEPES pH 7.5 was coupled to pre-washed N-hydroxysuccinimidyl-activated agarose beads (Sigma) at 4 C for 1 h. Remaining active sites were blocked by incubating in the presence of 0.1 mL 1 M ethanolamine pH 8 at 4 C for 1 h. Rat cultured mast cell line (RCMC [226]) was lysed with 1% triton X-100 in PBS with Complete Mini anti-protease cocktail tablets (Roche Applied Science, Laval, PQ, Canada). Supernatant remaining after 1000 g, 12,000 g, and 100,000 g centrifugations was loaded on columns. Columns were sequentially washed with 30 volumes lysis buffer, 20 volumes 10 mM Tris 0.5% triton X-100 300 mM NaCl pH 8, 20 volumes 10 mM sodium phosphate 0.5% triton X-100 450 mM NaCl pH 10, and eluted with 0.05 M diethylamine 0.5% triton X-100 650 mM NaCl pH 11.5. 1.5 mL fractions were collected into 50 µL 1 M Tris HCl pH 6.7.

2.2.8. MHC class I binding

PE labeled HLA-A*0201 tetramers assembled as described were a gift of Dr. John Elliott [71] (University of Alberta). Two peptides selected by the SYFPEITHI search engine, *Mycobacterium tuberculosis* antigen 85-B 143-152 (KLVANNTRL) and a diabetes-specific epitope of glutamic acid decarboxylase 114-123 (VMNILLQYVV) were assembled with HLA-A*0201. All washes and incubations of cells were done in ice cold PBS with 0.02% NaN_3. 1 x 10^6 human PBMC were incubated 15 min with 1.5 μg tetramers before the addition of CD14-FITC for 15 min. Before flow cytometry analysis cells were washed three times. In experiments where efforts were made to inhibit tetramer binding, cells were incubated with 40 μg/mL anti-CD8α mAb or isotype control for 30 min prior to addition of tetramers. MAb was not removed before incubation with tetramers.

Affinity purified (OX18) rat MHC class I was diluted in PBS to add 0.1 μg protein to each well of a 96 well plate (Becton-Dickinson, 351172) and allowed to adhere for 2 h at room temperature. Wells were subsequently incubated with 10% BSA in PBS for 30 min and washed three times with PBS. BSA binds macrophages at near background levels and provided reproducibly low background binding in our assay in comparison to other blocking substances tested (FBS, milk, unblocked plates). NR8383 rat AM (ATCC [227]) were labeled with the fluorescent dye calcein-AM (Molecular Probes) as previously described [72] and added to wells in PBS. Cells were centrifuged at 5 g for 3 min and incubated for 1 h at 37 C in 5% CO_2. Fluorescence remaining after six washes (PBS) was measured using a Millipore Cytofluor 2350 plate reader. All tests were done in triplicate in each experiment. Mean fluorescence of triplicate wells was calculated and percent binding was calculated for each experiment as mean fluorescence treated/mean fluorescence control treated x 100%.

2.2.9. Immune-Complex Stimulation of Monocytes and TNF Measurements

Monocytes were treated with isotype or anti-CD8α mAb (10 μg/mL), or immune-complexes. Immune-complexes were prepared by combining isotype mAb or anti-CD8α mAb (10 μg/mL) with anti-mouse Ig (20 μg/mL) for 15 min before addition to monocytes. Monocytes enriched on a Percoll gradient were incubated with immune-complexes for 5 h at 0.2 x 10^6 cells/well of a 96 well plate (Becton Dickinson, 35172). Anti-human TNF mAb, fixation and permeabilization buffers, and monensin were from ebioscience (San Diego, CA). Intracellular TNF was detected according to supplier recommendations. Monensin (2 μM) was added 2 h after immune-complexes. At 5 h, cells were fixed (4% paraformaldehyde, 20 min, room temperature), stained with anti-CD14-FITC, permeabilized (0.1% saponin) and stained with anti-TNF-PE for flow cytometry analysis.

Stimulation of monocytes with immune-complexes was inhibited by pretreating monocytes with 50 μg/mL purified mouse IgG Fc fragment (Jackson Immunoresearch, West Grove, USA) to block binding of immune-complexes to FcR. In these experiments free binding sites of anti-mouse Ig antibody in immune-complexes that might otherwise bind Fc fragments on pretreated monocytes, were pre-blocked with 50 μg/mL purified Fc fragment.

TNF release was measured by ELISA after activation of monocytes for 18 h. Cells were centrifuged (500 g, 3 min) and 150 μL of 200 μL supernatant was removed. Nunc MaxiSorp 96 well plates were coated overnight (4 C) with 2 μg/mL anti-human TNF mAb (clone 28401, R&D Systems, Minneapolis, MN) in 0.1M Na_2HPO_4 pH 9.0. Wells were blocked with 10% FBS in PBS for 1.5 h at room temperature and washed. All washing steps included 6 washes with excess PBS 0.05% Tween-20. Samples and recombinant human TNF standards (diluted in 10% FBS, R&D Systems) were added and incubated overnight at 4 C. After washing biotinylated anti-human TNF (BAF210, R&D systems) was added at 200 ng/mL for 1.5 h at room temperature. Wells were washed and streptavidin-HRP (1/5000 in 10% FBS, Vector Labs, Burlingame, CA) was added for 1.5 h at room temperature. Wells were washed and *o*-phenylenediamine substrate was added. Reactions were stopped with 2N H_2SO_4 and color was read at 490 nm using a PowerWave XS plate reader (Bio-TeK, Winooski, VT). Standard curves and concentrations were determined using the KC4 program (Bio-Tek).

2.2.10. LAT Phosphorylation

Three minutes after addition of immune-complexes monocytes were fixed and permeabilized according to manufacturer's instructions, with buffers adapted to measuring phosphorylation events by intracellular flow cytometry in human PBMC (BD Cytofix Buffer, BD Phosflow Permeabilization Buffer III, BD Biosciences). Non-specific binding was blocked with 5% milk in PBS. Phosphorylated residue Y132 of LAT was detected with rabbit polyclonal antibody (10 µg/mL, abcam, Cambridge, USA) followed by sheep anti-rabbit Ig-FITC (1/50, STAR34B, Serotec). Binding of phosphor-LAT Ab was compared to binding of rabbit Ig (Sigma) for each monocyte treatment. For western blot cells were lysed in pervanadate containing buffer (Cell Signaling Technologies, Danvers, USA). Equal amounts of protein were loaded after quantitation with the detergent-tolerant Bio-Rad RC DC protein assay system. Blots were blocked with 3% BSA (Sigma). Anti-LAT phospho-Y132 antibody (above) was used at 1 µg/mL.

2.2.11. Measurement of CD14 and CD69 Expression

After 18 h activation cells were stained with CD14-FITC/CD69-PE or isotype controls and fixed (ebioscience fixation buffer) before analysis on a FACScan.

2.3. Results

2.3.1. CD8α and not CD8β is present on human peripheral blood monocytes

Using six anti-CD8α mAb, we performed two-color flow cytometry (CD8α/CD14) on human peripheral blood mononuclear cells (PBMC) (>95% viable, Figure 2-1A-D). Monocytes were gated for analysis by expression of high levels of CD14 [228] and characteristic FSC/SSC scatter (Figure 2-1A). Several anti-CD8α mAb defined by human leukocyte differentiation antigen (HLDA) workshops and used for clinical diagnostic services were used, eg., B9.11, OKT8, 51.1, and LT8. Among lymphocytes (FSC/SSC gated) a subpopulation expressed high levels of CD8α and CD8β (Figure 2-1B, anti-CD8α mAb OKT8 and anti-CD8β-dependent mAb 2ST8.5H7 are shown as representative examples). In addition to the expected population of CD8α and CD8β + T cells, we detected moderate levels of CD8α on monocytes (Figure 2-1C, >3x geometric mean of isotype mAb). CD8β was not detected on monocytes with mAb 2ST8.5H7 (Figure 2-1C) or 5F2 (not shown), suggesting they do not express CD8αβ (mAb 2ST8.5H7) or putative CD8ββ dimers (mAb 5F2) [229]. Accordingly, mRNA for CD8β was detected in total PBMC but not in highly enriched monocytes (Figure 2-1C).

We investigated whether non-specific binding of mAb to Fc receptors contributed to binding of anti-CD8α mAb to monocytes. CD64 binds immunoglobulin with 1000-fold the affinity of other FcγR [230], and preferentially binds mouse IgG2a antibodies compared to mouse IgG1 [231]. Thus because most or all human monocytes express significant amounts of CD64 [228] mouse IgG2a has a 100-1000 fold higher affinity for binding human monocytes than mouse IgG1 [232]. Incubating monocytes with human Ig preparations [233] (not shown) or a mAb which blocks binding of Ig to CD64 (clone 10.1) [225] slightly decreased binding of isotype mAb (11-27% geometric mean) and did not decrease binding of anti-CD8α mAb (Figure 2-1D), suggesting that binding of CD8α mAb to monocytes is specific for CD8α and that basic blocking steps used (e.g. 5% milk, 0.1% BSA) adequately minimized non-specific mAb binding, such as to Fc receptors. The monocytic cell line THP-1 also bound CD8α mAb, (Figure 2-1E) at moderate levels comparable to blood monocytes (Figure 2-1C). We conclude that moderate levels of CD8α are found on human monocytes.

2.3.2. CD8α on human monocytes: confocal microscopy

The adhesion of T cells and monocytes to each other may misleadingly be read as individual cells co-expressing CD14 and CD8. Thus to determine the localization of CD8α on monocytes we performed two-color confocal microscopy of permeabilized PBMC (Figure 2-2). All anti-CD8α mAb detected CD8α at the periphery of CD14^{hi} monocytes and some CD3^{hi} T cells (Figure 2-2B-E mAb B9.11 is shown, and is representative of results obtained with LT8, OKT8, 32-M4, 51.1, and Nu-Ts/c). CD8α was also observed intracellularly in some monocytes with a distribution resembling CD14, suggesting that similar to NK cells [234] CD8α may be found intracellularly in monocytes. CD8α+/CD14- cells (likely CD8α+ T cells) were observed alongside CD8α+/CD14+ monocytes (Figure 2-2E).

2.3.3. Human alveolar Mφ (AM) bind anti-CD8α mAb

To establish whether CD8α may be expressed by alveolar Mφ, bronchoalveolar lavage samples from five consecutive patients were obtained (two sarcoidosis, squamous cancer, bilateral infiltrates, pulmonary nodule). About one-third of AM from four of five patients (pulmonary nodules, two sarcoidosis, squamous cancer) bound anti-CD8α mAb at low levels

(Figure 2-3). In a fifth sample (bilateral infiltrates), AM were not recognized by anti-CD8α mAb (data not shown).

2.3.4. Human monocytic line THP-1 expresses CD8α mRNA

To determine if monocytes can synthesize CD8α, RT-PCR for CD8α was performed using commercial primers as published [235]. Due to the sensitivity of RT-PCR for mRNA from contaminating cells, we studied the cultured monocytic line THP-1 in addition to highly enriched monocytes (negative for T cell/NK cell specific CD3ζ mRNA, >99% FSC/SSC and CD14^{hi} monocytes, Figure 2-4A). CD8α mRNA was detected in peripheral blood mononuclear cells (containing CD8α+ T cells), THP-1 monocytic cell line, and highly purified blood monocytes, but not in a lung epithelial cell line (A549) as expected (Figure 2-4B). We conclude the monocytic cell line THP-1, and probably ex vivo monocytes synthesize CD8α mRNA.

2.3.5. Western blot analysis of CD8α in monocytes, Mφ and T cells

To test whether CD8α is detected in human monocytes by a method without possible contribution of FcγR we performed western blot for CD8α. Proteins at 32 kDa were detected with anti-CD8α mAb D9 by western blot (Figure 2-4C) of thymus lysate, blood lymphocytes, immature monocytes (THP-1), mature *ex vivo* monocytes (>99% CD14^{hi}, CD3ζ(-ve), enrichment Figure 2-4A), and Mφ differentiated with GM-CSF from blood monocytes, but not in lung epithelial cells (A549, negative control). Similarly, a 32 kDa protein was found by western blot with anti-CD8α mAb B9.11 in PBMC and THP-1 (Figure 2-4C).

2.3.6. Fewer Sialylated 34 kDa Versions of CD8α are found on Monocytes Compared to T cells

CD8α[236], like CD8β [237], is less sialylated on immature thymocytes than on mature thymocytes. This sialylation influences the ability of CD8 to bind MHC class I and induce T cell activation [238]. Sialic acid has a significant negative charge demonstrable by acidic shifts in isoelectric points (pI) in 2-D electrophoresis.

By western blot after 1-D SDS-PAGE in Figure 2-4C, it was not clear if anti-CD8α mAb detected more than one closely clustered Mr of CD8α. Other studies have found that CD8α is two Mr species of 32 and 34 kDa distinguished by glycosylation [239], and that CD8α from blood T cells has a 2-D electrophoretic pattern extending in discrete spots across basic pI ranges [193;240]. To better grasp the potential molecular differences between CD8α on monocytes and T cells we performed 2-D electrophoresis with a higher Mr resolution provided by 4-15% polyacrylamide gradient gels.

Polyclonal anti-CD8α antibody detected discrete spots across 2-D gels from the predicted pI of unglycosylated CD8α (~9) to pI 6-7, at Mr from 32-34 kDa (Figure 2-5). Many of the spots detected for CD8α at more acidic pI (6-8.5) are due to sialic acid modification of CD8α as neuramindase treatment of lymphocytes or monocytes eliminated several of the more acidic CD8α spots (pI 6-8.5), leaving three dominant spots at pI 8-9, two at 32 kDa and one at 34 kDa (Figure 2-5). Notably, monocytes had much less of the sialylated 34 kDa forms of CD8α than T cells in samples from three individuals (Figure 2-5).

2.3.7. CD8α on monocytes and Mφ binds MHC class I

CD8α on rat Mφ binds two (OX8, R1-10B5) of three (G28) CD8α-specific mAb [203] (unpublished data, Derrick Gibbings). The anti-CD8α mAb that does not bind rat Mφ (clone G28) binds the Ig-like MHC class I binding region of CD8α [241]. Thus CD8α on monocytes and macrophages may have a different structure or inter-molecular associations than CD8α on T cells, and may not bind MHC class I. We set out to test this possibility.

Thymocytes from CD8β knockout mice bind MHC class I tetramers and overexpression of CD8α enhances this CD8-dependent binding [242], suggesting that despite the heightened ability of CD8αβ (at least in an unsialylated form on thymocytes [237;238]) to bind MHC class I tetramers, CD8αα is fully capable in this regard. We studied whether monocytes, that seem to express CD8αα homodimers, bind tetramers of MHC class I via CD8α. Studies with CTL clones show that MHC class I tetramer binding may be unaffected, variably inhibited or enhanced by anti-CD8α mAb, depending on which anti-CD8α mAb is used, and TCR affinity [238;243;244]. We expected that anti-CD8α mAb would not block all tetramer binding to human monocytes because members of the immunoglobulin-like-transcript (ILT/CD85) family (ILT2, 4) expressed by monocytes bind MHC class I tetramers [245], interact with regions on MHC class I that overlap with CD8α and thus compete with CD8α for binding of MHC class I [246].

HLA-*0201 tetramers bound to nearly all CD14$^{\text{hi}}$ monocytes (Fig 6A). Tetramers complexed with two different peptides bound similarly to monocytes (data not shown). Others have found that anti-CD8α mAb B9.11 inhibits binding of HLA tetramers to T cells.

Here clone B9.11 inhibited HLA tetramer binding less than anti-CD8α mAb D9 (Figure 2-6B, 11.6% vs.18.6%, $p<0.05$). HLA tetramer binding was not affected by clone 32-M4 (despite its ability to bind CD8α on monocytes), or isotype control mAb (Figure 2-6B). The lack of effect of 32-M4 on tetramer binding demonstrates that binding mAb to the surface of monocytes does not non-specifically block tetramer binding. Thus, human monocytes appear to bind MHC class I through CD8α (current study), and as shown by others, through ILT2 and ILT4 [245].

To test whether CD8α on rat Mφ can bind MHC class I we used the rat AM cell line NR8383 that expresses CD8α [203] and affinity purified MHC class I. MHC class I was established to be highly enriched by silver staining, western blot (OX18), and MALDI-QTOF sequencing (data not shown) of purified fractions. Nineteen percent of NR8383 AM adhered to wells coated with BSA (Figure 2-6C). Significantly more NR8383 (28%, $p<0.05$) bound to MHC class I coated wells.

When binding of T cell clones to MHC class I is examined in a similar system ligation of TcR enhances binding of CD8 to MHC class I [247]. We hypothesized that the similar signaling mechanisms activated by FcγR may allow it to enhance CD8 binding to MHC class I, like TCR. We found that while isotype antibody had no effect on NR8383 binding to BSA, it significantly enhanced binding to MHC class I coated wells (38%, $p<0.05$). This suggests that binding immunoglobulin to Mφ, potentially through FcγR, enhances Mφ binding to MHC class I. Anti-CD8α mAb R1-10B5 had no effect on binding to BSA coated wells and thus did not inhibit AM binding in general, but reduced binding of NR8383 to MHC class I coated wells to a level equivalent to binding of BSA (18%, $p<0.05$; Fig 6C). Thus, the potentially novel structure of rat Mφ CD8α does not interfere with its ability to bind MHC class I.

2.3.8. Anti-CD8α mAb amplifies monocyte responses to immune-complexes through FcγR

TCR and FcR use analogous and sometimes interchangeable signaling mechanisms to activate cells [204;248]. We investigated if CD8α on monocytes might be involved in responses to immune-complexes mediated by FcR. When others have screened several anti-CD8α mAb for effects on CTL cytotoxicity the effect has ranged from substantial inhibition to no effect [249]. Thus, we used a panel of anti-CD8α mAb[250;251]. Anti-CD8α mAb segregate into two classes that affect either MHC class I binding or transmembrane signaling, but not both [252]. Thus we anticipated that anti-CD8α mAb that blocked MHC class I binding might not activate monocytes.

When monocytes were stimulated with preformed immune-complexes (isotype mAb-anti-mouse Ig), CD14^{hi} monocytes produced moderate amounts of intracellular TNF (Figure 2-7A, flow cytometry histogram IgG$_{2a}$ immune-complex). Additional monocyte stimulation was added through CD8α by using an immune-complex formed with anti-CD8α mAb 32-M4 (32-M4-anti-mouse Ig). With this further stimulation through CD8α, monocytes produced 2-fold more intracellular TNF than monocytes treated with isotype immune-complexes alone (Figure 2-7A, 32-M4 immune-complex histogram). In contrast to immune-complexes containing anti-CD8α mAb 32-M4, immune-complexes containing anti-CD8α mAb D9 induced a trend toward enhanced TNF production that did not reach statistical significance.

To evaluate whether monocyte activation by immune-complexes occurred in a FcγR-dependent manner, monocytes were pretreated with Fc fragment of Ig (50 μg/mL) to compete with immune-complexes for binding to FcR. Immune-complexes were also pretreated with Fc fragments to block free sites on anti-mouse Ig cross-linking antibody that may bind Fc fragments on pretreated monocytes. Blockade of FcR with excess Fc fragments nullified TNF release instigated by isotype and anti-CD8α immune-complexes (Figure 2-7A). Thus, as anticipated monocyte activation with isotype containing immune-complexes was probably mediated by receptors for the Fc fragment of Ig (FcR).

Treatment of monocytes with monomeric non-specific IgG_{2a} mAb induced a slight increase in monocyte TNF production (Figure 2-7A, flow cytometry histogram $IgG_{2a)}$. This is likely due to the ability of the high affinity FcγR, CD64, found on the majority of monocytes to bind monomeric mouse IgG_{2a} [228;232;253]. Monomeric anti-CD8α mAb did not increase monocyte TNF production more than control IgG_{2a} mAb (Figure 2-7A, flow cytometry histogram $IgG_{2a)}$. As CD8α enhancement of TNF production is inhibited by Fc fragments and does not occur with monomeric anti-CD8α mAb, the ability of anti-CD8α mAb to enhance responses of human monocytes appears to depend on co-engagement of FcR.

To evaluate whether intracellular TNF is secreted following monocyte activation in our system TNF ELISA was performed on supernatants (Figure 2-7B). In agreement with intracellular flow cytometry, immune-complexes containing anti-CD8α mAb 32-M4 induced release of approximately 3-fold more TNF than isotype immune-complex alone (1376 pg/mL versus 468 pg/mL, $p < 0.05$), and immune-complexes containing anti-CD8α mAb D9 induced a trend toward heightened TNF production that did not reach statistical significance. Immune-complexes containing anti-CD8α mAb OKT8 did not significantly increase monocyte TNF production above control immune-complexes, indicating as shown by others [228]that monocyte TNF release is not stimulated by any immune-complex containing a mAb that binds to the monocyte surface.

CD8α associates with LAT, and stimulation of CD8α on T cells results in increased LAT phosphorylation [194]. LAT is also phosphorylated and functionally important in responses of receptors that signal through the common γ chain like FcεRI , FcRI, FcRIIa[254;255]. RT-PCR confirmed that highly enriched human monocytes (see Figure 2-4A) contain mRNA for LAT (Figure 2-7C). We examined the phosphorylation status of a key tyrosine (Y132) involved in LAT recruitment of PLCγ1 and Ca+2 activation [256;257] after stimulation of monocytes through anti-CD8α containing immune-complexes. Increased phosphorylated LAT-Y132 was detected by intracellular flow cytometry after stimulation of monocytes with immune-complexes, and phosphorylation was further increased after additional activation through CD8α (Figure 2-7C, left). Western blot confirmed increased phosphorylation of LAT-Y132 after stimulation of monocytes through CD8α compared to isotype immune-complexes (Figure 2-7C, right). This suggests that monocyte activation through CD8α increases LAT phosphorylation.

Monocytes activated through immune-complexes and CD8α were analyzed for markers of monocyte activation and maturation. CD69 is constitutively expressed by human monocytes

[258], but is upregulated after stimulation [232]. Stimulation of monocytes through immune-complexes and anti-CD8α mAb 32-M4 induced an increased level of CD69 expression on 2.3-fold more monocytes than immune-complexes alone (Figure 2-7D bar graph, $p<0.05$, flow cytometry density plot is one representative example of four experiments). CD14 is downregulated during monocyte maturation into macrophages and dendritic cells [259]. Stimulation of monocytes through immune-complexes and anti-CD8α mAb 32-M4 resulted in decreased CD14 expression (54.5% geometric mean, $p<0.05$) on monocytes compared to immune-complexes alone (Figure 2-7D). While immune-complexes containing anti-CD8α mAb D9 induced a trend toward heightened TNF production that did not reach statistical significance, D9 containing immune-complexes did significantly decrease CD14 expression (Figure 2-7D). Thus, monocytes stimulated through CD8α in addition to immune-complexes induce increased monocyte activation and maturation, TNF release, and LAT phosphorylation.

2.4. Discussion

We have identified the expression of CD8α by human monocytes and confirmed that CD8α on monocytes binds MHC class I. Whatsmore, we demonstrate that monocyte activation via CD8α amplifies responses to immune-complexes several-fold, in a manner inhibitable by Fc fragments.

The presence of small percentages (1-20%) of CD14+/CD8α^{hi} monocytes in patients, some with viral infection, (e.g. [206;209]) may be subject to several interpretations besides the expression of high levels of CD8α by a few monocytes. Human PBMC from untreated HIV patients or activated in vitro have small percentages of CD14+/CD8α^{hi} cells, but this population is due to acquisition of CD14 from monocytes, by T cells, that are mostly CD8α+ [260;261]. In our studies, a small population (4-8%) of gated monocytes appeared to have high levels of CD8α in Figure 2-1C. However, CD8α^{hi} monocytes were not observed using directly-conjugated anti-CD8α mAb-PE, (Figure 2-1D). After flow cytometry sorting and re-analysis of putative CD8α^{hi} monocytes (Figure 2-1C) large proportions of CD14-/CD8α^{hi} and CD14^{hi}/CD8α^{low} cells were observed suggesting that putative CD8α^{hi} monocytes (Figure 2-1C) were likely due to doublets of CD8+ T cells aggregated with monocytes. Thus, previous to the present study no evidence existed to demonstrate that human monocytes synthesized CD8α or that CD8α potentially found on these cells was functional.

We provide strong evidence through several techniques that human monocytes constitutively express CD8α at low levels. Several anti-CD8α mAb bound human monocytes, and blockade of background FcR binding mediated by CD64 enhanced the staining of CD8α on monocytes compared to isotype control (Figure 2-1D). In addition, CD8α was observed in monocytes, and the monocytic cell line THP-1 by western blot confirming the presence of CD8α in these cells without possible contribution of FcγR to binding of anti-CD8α mAb. As the secreted form of CD8α is 27 kDa, the 32 and 34 kDa forms of CD8α we detected in monocytes are not likely due to adsorption from alternatively spliced and secreted CD8α [239]. Finally, detection of CD8α mRNA in THP-1, CD8α protein on THP-1 and 32 kDa CD8α in lysate of continuously cultured THP-1, demonstrates that these monocytic cells must transcribe and translate CD8α Although we have identified CD8β on rat alveolar Mφ [203] and mast cells [202], and others have found CD8β on rat monocytes [206] and γδ T cells [262] we did not detect CD8β protein or mRNA in human monocytes. Expression of CD8β on monocytes and Mϕ may be restricted to rats.

T cells/dendritic cells express CD8α in all mammals tested [200;200;263], whereas CD8α is expressed by NK cells [201;205] and monocytes/ Mφ [203] in humans and rats, but not mice [207;208]. Similarly CD4 is expressed by human but not mouse macrophages [264]. Thus, the expression of CD8 and CD4 on monocytes and macrophages occurs in humans and rats, but apparently not mice. Distinct regulatory elements control expression of the human CD4 gene in T cells/dendritic cells compared to Mφ [265]. Changes across species in Mφ-specific CD4 and CD8 regulatory elements may enable CD4 and CD8 to be expressed on monocytes and Mφ only in rats and humans but not mice.

Monocytes lack 34 kDa sialylated forms of CD8α compared to T cells. The Mr difference between 32 and 34 kDa forms of CD8α may be due to glycosylation other than sialylation [239] (Figure 2-5), or palmitoylation of CD8α [266;267] at three eligible membrane-proximal cysteines [268;269]. Monocytes and T cells may express different amounts or kinds of glycosylation or palmitoylation enzymes that account for selective accumulation of 32 or 34 kDa CD8α. Differential glycosylation and palmitoylation of CD8α are known to enhance or inhibit its ability to bind MHC class I and induce T cell activation[237;238;270;271]. Phosphorylation of CD8α, or oxidation of the free cysteine [272] in the CD8α Ig-domain may cause the difference in pI of the forms of CD8α remaining after neuraminidase treatment.

As anti-TCR mAb vary widely in their ability to induce T cell activation depending on the epitope recognized [273], when others have used several anti-CD8α mAb were used to examine CD8 binding to MHC class I or effect on CTL cytotoxicity some anti-CD8α mAb had no effect, others significantly enhanced effects, and others substantially inhibited [237;243]. Similarly, in our studies only certain anti-CD8α mAb inhibited MHC class I binding or activated monocytes. Others have found that anti-CD8 mAb segregate into those that block MHC class I binding and those that affect cell activation [252]. Mirroring this, anti-CD8α mAb used here segregate somewhat into those that block MHC class I binding (D9, B9.11), and those that induce monocyte activation (32-M4, D9).

Inhibition of MHC class I tetramer binding by T cells with anti-CD8α mAb requires co-ordinate binding of TCR [237;238;243;274], except at one stage of mouse thymic selection when sialylation of CD8β allows non-cognate binding of CD8 to tetramers in the absence of TCR [238] through affinity or avidity enhancement. Our data suggests CD8α on monocytes can, as occurs with CD8α on immature mouse thymic T cells, bind tetramers in a non-cognate manner, ie., without TCR-peptide interactions. This suggests (a) CD8α on monocytes independently binds MHC class I and has a higher affinity for MHC class I than CD8α on blood T cells and T cell clones tested; (b) CD8α stabilizes the interaction of a receptor other than TCR with MHC class I; or (c) CD8α binds MHC class I independently after the "triggering" of CD8α avidity-enhancement through a receptor sharing signaling mechanisms with TCR. CD8α-dependent tetramer binding found here likely does not involve "triggered" binding, stimulated through a receptor sharing TCR signaling mechanisms because this requires cytoskeletal re-organization [247], and in our studies cells were kept on ice with sodium azide.

As CD8α enhancement of TNF production is inhibited by Fc fragments and does not occur with monomeric anti-CD8α mAb, the ability of anti-CD8α mAb to enhance responses of human monocytes appears to depend on co-engagement of FcR. Many of the components of FcR and TCR signaling are conserved, homologous or interchangeable, such as Fcγ and CD3ζ chain, ZAP-70 and Syk, or LAT. Fcγ and CD3ζ are conserved ancestral duplicates [275;276] that can substitute for each other in activation of γδ or αβ T cells [204;248;277]. In addition, CD3ζ and Fcγ both function with FcR [278], NKp30, and NKp46 [279] in NK cells, and FcεRI in mast cells [280]. We previously demonstrated that CD8α on rat Mφ signals through Syk and src tyrosine kinases [219;281;282]. In addition to its involvement in TCR signaling, LAT has previously been shown to be involved in FcR-mediated signaling of human monocytes [254]. Here we demonstrate that CD8α enhances immune-complex-mediated phosphorylation of LAT. Thus CD8 signaling on monocytes, likely with FcR, enhances LAT phosphorylation, like CD8 signaling with TCR on T cells enhances LAT phosphorylation.

CD8 enhances T cell IL-2 production 2-9 fold when it does not bind the same MHC class I that activates TCR [195-197]. In quantitative agreement, we found that co-ligation of FcR and CD8 enhanced monocyte TNF release 3-fold. This suggests that CD8α can enhance activation of monocytes or T cells independent of its co-receptor activity, defined as the ability of CD8α to bind the same MHC class I molecule as its partnered receptor, such as TCR. If this is the case, CD8α may be able to enhance responses of NK cells, dendritic cells, mast cells, monocytes and macrophages through receptors utilizing the γ chain or CD3ζ, like FcR, ILT1 [283], Nkp30 and Nkp46.

Our evidence suggests monocyte adhesion and responses instigated through immune-complexes and FcR can be amplified by CD8α. Interestingly, in rats CD8α+ monocytes and Mφ are found at sites of tissue damage in immune-complex mediated glomerulonephritis [215], arthritis [213], experimental allergic encephalomyelitis (a model of multiple sclerosis) [284], and ischaemia-reperfusion injury [285]. TNF is an important mediator in many of these diseases [217;218;286;287]. As monocyte CD8α interacts with MHC class I, and TNF production is enhanced by immune-complex/CD8α activation, CD8α on monocytes may enhance TNF production in some autoimmune and acute inflammatory conditions characterized by deposition of immune-complexes on MHC class I expressing tissues. The possible contribution of CD8+ monocytes to responses thought to be mediated by CD8+ T cells in rats and humans would be excluded from mouse models where monocytes and Mφ do not appear to express CD8.

In summary, we find that human monocytes express CD8α differentiable from that on T cells by 2-D electrophoresis. We provide evidence that CD8α on monocytes amplifies pro-inflammatory responses initiated through FcR, suggesting for the first time a co-activator role for CD8 on cells without the TCR. This study suggests CD8α on monocytes is potentially culpable in the pathology of arthritis, ischemia, multiple sclerosis and other immune-complex/FcR mediated diseases.

2.5. Figure Legends

Figure 2-1. CD8α is detected by flow cytometry on CD14^{hi} monocytes from human peripheral blood.

Anti-CD8α mAb bind monocytes by flow cytometry. (A) Monocytes were gated by characteristic FSC/SSC scatter (left panel) and expression of high levels of CD14 (right panel). (B) A population of lymphocytes, gated by characteristic FSC/SSC scatter, bind high levels of anti-CD8α and anti-CD8β mAb. (C) Blood monocytes gated in (A) bind anti-CD8α mAb, but not CD8β mAb. Monocytes enriched to greater than 99% did not contain CD8β mRNA. Flow cytometry markers shown are identical for all antibodies of similar isotype within each experiment (LT8, B9.11 are IgG1; others are IgG2a). M1, M2 denote designated markers. Markers defining subpopulations of cells were generated to compare fluorescence associated with the CD8α^{low} population versus isotype negative control (M2), and to determine the percentage of monocytes apparently binding high levels of anti-CD8α mAb (M1). M2: number is the geometric mean of fluorescence of cells within subpopulation marked (WinMDI). M1: % is the percentage of cells within subpopulation M1 (apparent CD8α^{hi} monocytes, subsequent investigation suggested this population is not monocytes, see text). Results are representative of five experiments. (D) Blockade of Ig binding to CD64 with anti-CD64 mAb does not markedly decrease binding of isotype mAb or anti-CD8α mAb to monocytes beyond that seen by blocking with 5% milk. Bracketed numbers are geometric means of fluorescence of indicated peaks. (E). The monocytic cell line THP-1 is bound by anti-CD8α mAb. B9.11 is representative of other anti-CD8α mAb (LT8, 32-M4) and three independent experiments.

Figure 2-2. CD8α is detected by confocal microscopy on peripheral blood monocytes and lymphocytes with several anti-CD8α mAb.

(A) CD3-FITC and CD14-FITC bind PBMC in contrast to isotype control. (B-E) Anti-CD8α mAb binds monocytes and lymphocytes in contrast to isotype control. Isotype antibody (B, C) and anti-CD8α mAb B9.11 (D, E) were detected with anti-mouse Ig-Rhodamine Red (RR, red channel, right panel). After incubation with normal mouse serum monocytes and lymphocytes were labeled with anti-CD14-FITC (C, E)) or anti-CD3-FITC (B, D) shown in the green channel, left panel. Results are representative of several anti-CD8α mAb (OKT8, 51.1, 32-M4, Nu-Ts/c, and B9.11) in three experiments.

Figure 2-3. CD8α is detected by flow cytometry on AM from human bronchoalveolar lavage.

Anti-CD8α mAb bound human AM from four of five consecutive patients. Shown are results from a patient with pulmonary nodules and CD8α+ AM. Markers defining populations of cells are identical for all antibodies of similar isotype within each experiment (LT8 is IgG1; others are IgG2a). Number X GM designates fold increase in geometric mean of fluorescence of gated AM stained with anti-CD8α mAb compared to appropriate isotype mAb. Percentages represent proportion of CD8α+ AM calculated using markers (M1) shown. AM were gated as described [288].

Figure 2-4. Human monocytes express CD8α mRNA and CD8α protein.

(A) Left, purification of monocytes was confirmed by flow cytometry analysis. FSC-SSC scatter plot (left) and CD14 histogram (right) show a homogenous population of 99% CD14^{hi} monocytes. Percentages of cells within gates are shown below. Right, purification of monocytes was also confirmed by attempting to detect the T and NK cell transcript, CD3ζ, in monocytes. RT-PCR for a 293 bp fragment of CD3ζ mRNA was performed with 50 cycles of amplification. CD3ζ mRNA was detected in PBMC, containing T cells, but not monocytes, the monocytic line THP-1, or lung epithelial cells (A549) (B) A 379 bp CD8α mRNA fragment was detected by RT-PCR in THP-1, >99% CD14^{hi} monocytes, PBMC, but not in a lung epithelial cell line, (A549) using intron-spanning primers, and 35 cycles of cDNA amplification. Detection of *β*-actin mRNA confirmed RNA extraction and RT-PCR was performed successfully. Results are representative of four separate experiments. (C). Left, western blot with anti-CD8α D9 detects a 32 kDa protein as expected for CD8α in THP-1, peripheral blood lymphocytes, and thymus lysate, but not in the lung epithelial line A549 (CD8α negative control). Right, anti-CD8α mAb B9.11 detects a 32 kDa protein as expected for CD8α in THP-1 and peripheral blood lymphocytes. Bottom, western blot with D9 detects CD8α at indistinguishable molecular masses in THP-1, peripheral blood monocytes (>99%), GM-CSF differentiated macrophages, PBMC, thymus, and a CTL clone. 1-1.5 x 10^6 cell equivalents were loaded in each lane. Results are representative of four experiments.

Figure 2-5. Sialylated versions of CD8α at 32 and 34 kDa differentiate monocytes and T cells.

Western blot with a polyclonal anti-CD8α antibody after 2-D electrophoresis of *ex vivo* monocytes and lymphocytes. Cell lysates of lymphocytes and monocytes from one donor were separated by adherence, halved and treated (bottom panels) or not treated (top panels) with neuraminidase before analysis. Results are representative of experiments with cells from three donors.

Figure 2-6. CD8α on human peripheral blood monocytes and rat AM binds MHC class I.

(A). Binding of MHC class I tetramers to $CD14^{hi}$ monocytes is partially blocked by anti-CD8α mAb. Binding of 1.5 μg phycoerythrin labelled MHC class I tetramers to PBMC was measured by flow cytometry. To inhibit tetramer binding cells were pretreated with 40 μg/mL anti-CD8α mAb or isotype control for 30 min. Dark histogram is background fluorescence of $CD14^{hi}$ monocytes, light histogram represents binding of PE-labeled tetramers. (B) Bar graph is pooled results of gated $CD14^{hi}$ monocytes from three different donors in separate experiments. Inhibition of tetramer binding is expressed as a percent of binding (percent decrease in mean fluorescence intensity) after treatment with anti-CD8α mAb compared to matched isotype mAb (* $p<0.05$, non-paired t-test). (C). Adherence of NR8383 AM to plate-bound MHC class I is enhanced by immunoglobulin and inhibited by anti-CD8α mAb. NR8383 labeled with calcein-AM were allowed to adhere to MHC class I or BSA (open bars) alone or in the presence of 20 μg/mL isotype antibody (grey bars) or anti-CD8α mAb R1-10B5 (black bars). Results are pooled from three experiments. *: $p<0.05$ non-paired t-test, ns: not significant, $p>0.05$.

Figure 2-7. CD8α enhances monocyte TNF release, LAT phosphorylation, maturation and activation in a CD8α and Fc dependent manner.

Monocytes were stimulated with immune-complexes, preformed with anti-CD8α mAb or non-specific isotype mAb (10 μg/mL) cross-linked with anti-mouse Ig (20 μg/mL). (A) TNF production was measured by intracellular flow cytometry after 5 h. Histograms are representative results gated on $CD14^{hi}$ monocytes. The transition from a population of monocytes producing mid-levels of TNF after stimulation with isotype immune-complexes, to all monocytes producing high levels of TNF after CD8-immune-complex activation (32-M4) can be observed. Monocytes were also treated with monomeric mAb, or excess Fc fragment in some experiments to suggest the FcR-dependence of monocyte responses. Bar graph represents average geometric mean of intracellular TNF detected in monocytes from four separate experiments with different donors. Standard error of the mean is designated by error bars. *: $p<0.05$ non-paired t-test. (B) TNF release from monocytes activated as in (A) was measured at 18 h. Results are pooled from three donors, * $p<0.05$. (C) Phosphorylation of LAT Y132 is increased in a CD8α dependent manner. Left, intracellular flow cytometry measurement of LAT phosphorylation. Bar graph quantifies increased phosphorylation of LAT Y132 (LAT-P) compared to rabbit Ig control for each monocyte treatment in four donors using the following calculation: Geometric mean (Gm) (Gm LAT-P – Gm rabbit Ig among CD8α-IC activated cells) / (Gm LAT-P – Gm rabbit Ig among IC activated cells) x 100. Error bars show standard error of the mean. * $p<0.05$. Right, increased phosphorylation of LAT Y132 was confirmed by western blot detection of a 38 kDa band with anti-phospho-LAT-Y132 antibody. RT-PCR for LAT confirmed ability of monocytes to express LAT. (D) Expression of CD14 and CD69 was measured on monocytes activated for 18 h as in (A) to indicate their activation and maturation. Monocytes were stained with CD14-FITC/CD69-PE (above) or IgG2a-FITC/IgG2a-PE (below). Shown is a representative result. Numbers indicate percent cells above line-marker of CD69^{hi} expression. CD14^{hi} cells decrease and CD69^{hi} cells increase after activation with CD8α + immune-complex (32-M4 IC) versus immune-complex alone (IgG2a-IC). Bar graphs (right) are pooled data from three donors. Percentage increase in CD69^{hi} cells was measured as (%CD69^{hi} - % isotype control among CD8α IC activated cells) / (%CD69^{hi} - % isotype control among isotype IC activated cells) x 100. Decrease in intensity of CD14 staining was measured as (geometric mean (GM) CD14 – GM isotype among CD8α IC activated cells) / (GM CD14 – GM isotype IC among activated cells) x 100. * $p < 0.05$.

2.6. Figures

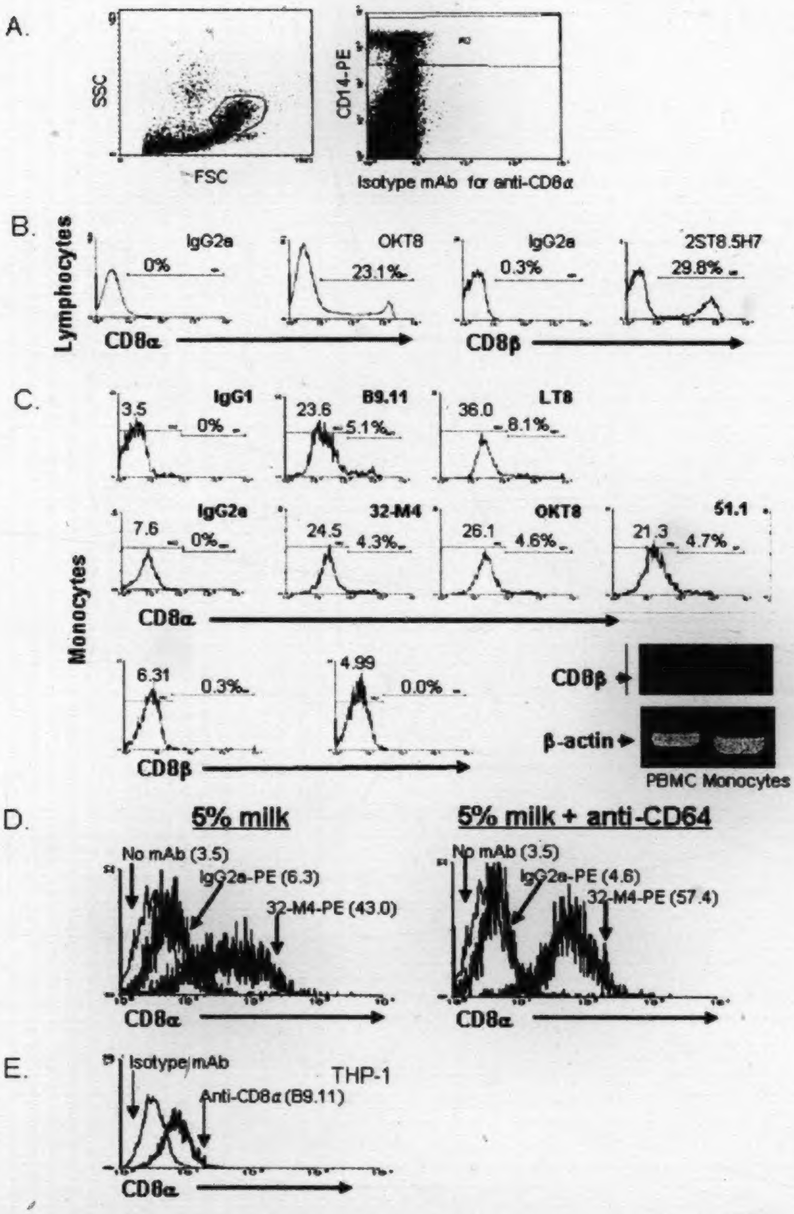


Figure 2-1. CD8α is detected by flow cytometry on CD14hi monocytes from human peripheral blood.

Figure 2-2. CD8α is detected by confocal microscopy on peripheral blood monocytes and lymphocytes with several anti-CD8α mAb

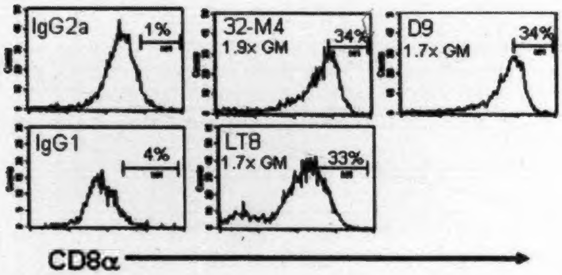
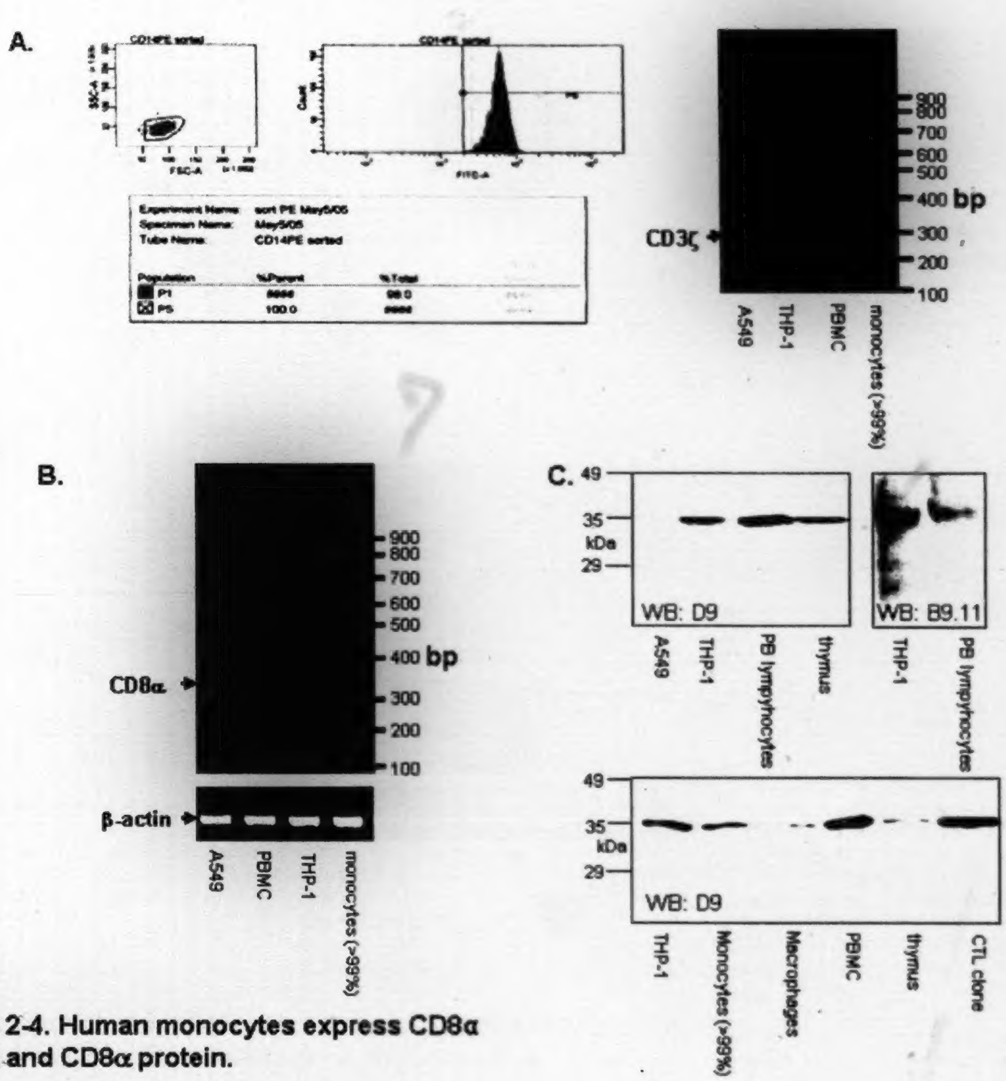


Figure 2-3. CD8α is detected by flow cytometry on AM from human bronchoalveolar lavage.


A.
CD14PE sorted
SSC-A
FSC-A
CD14PE sorted
Count
FITC-A
P5
CD3ζ
900
800
700
600
500
400 bp
300
200
100
A549
THP-1
PBMC
monocytes (>99%)
B.
CD8α
900
800
700
600
500
400 bp
300
200
100
β-actin
A549
PBMC
THP-1
monocytes (>99%)
C.
49
35
kDa
29
WB: D9
A549
THP-1
PB lymphocytes
thymus
WB: B9.11
THP-1
PB lymphocytes
49
35
kDa
29
WB: D9
THP-1
Monocytes (>99%)
Macrophages
PBMC
thymus
CTL clone

Experiment Name: sort PE May5/05
Specimen Name: May5/05
Tube Name: CD14PE sorted

Population	%Parent	%Total
P1	####	98.0
P5	100.0	####

Figure 2-4. Human monocytes express CD8α mRNA and CD8α protein.

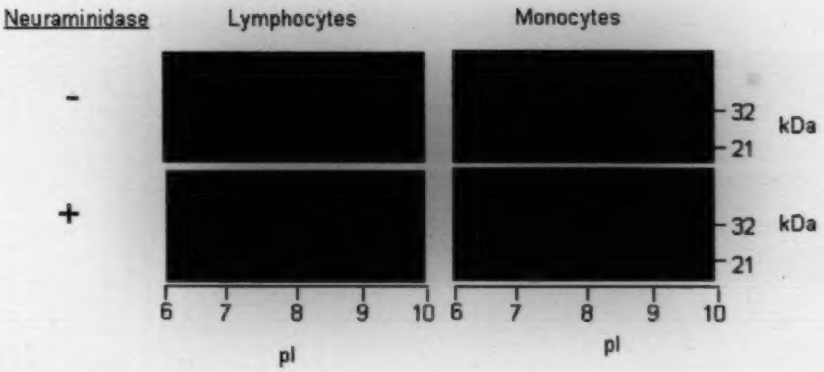

Neuraminidase
Lymphocytes
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Figure 2-5. Sialylated versions of CD8α at 32 and 34 kDa differentiate monocytes and T cells.

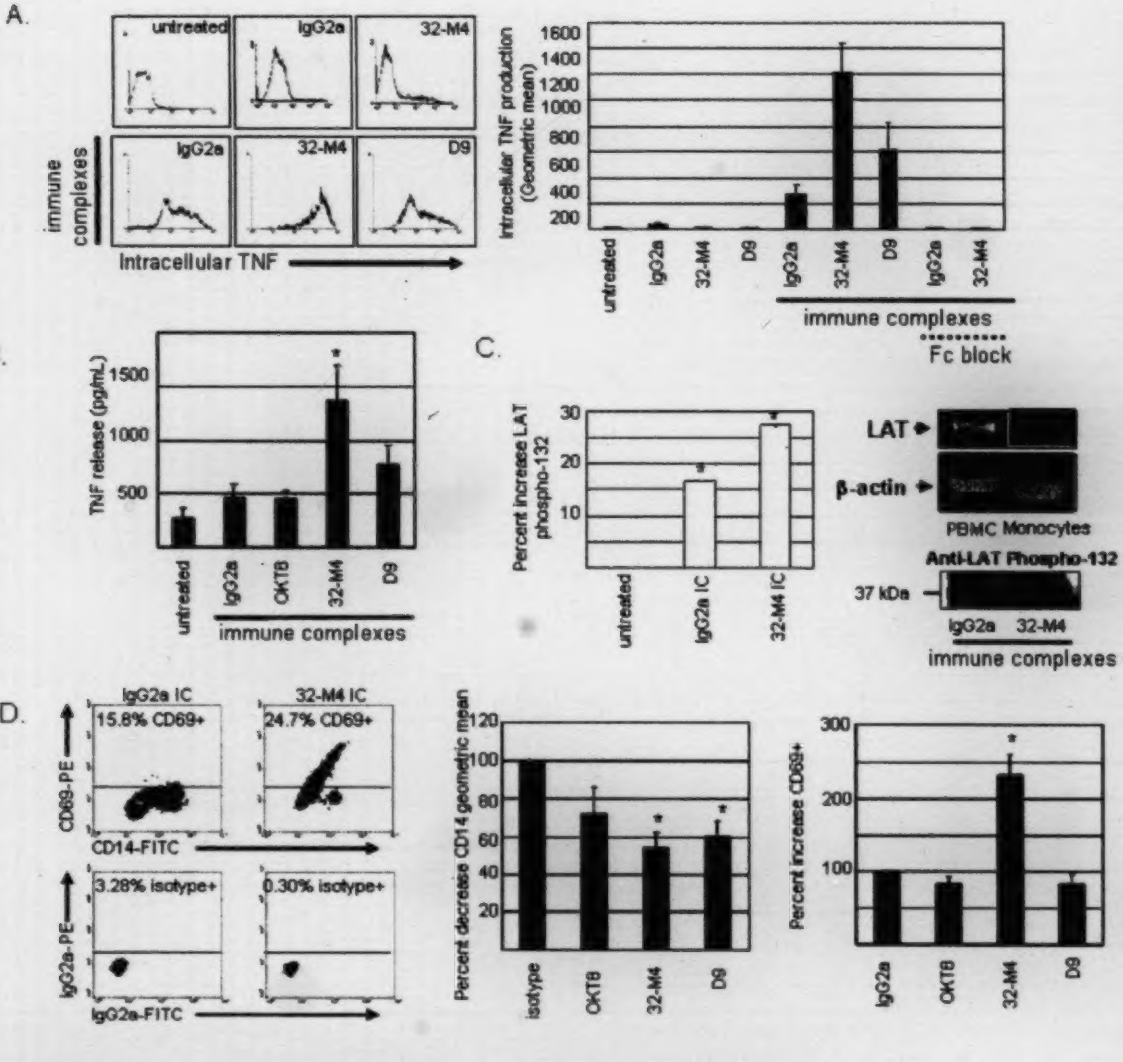

Figure 2-7. CD8α enhances monocyte TNF release, LAT phosphorylation, maturation and activation in a CD8α and Fc dependent manner.

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3. ANTI-CD8α MONOCLONAL ANTIBODY D9 BINDS CD8α ON HUMAN MONOCYTES AND T CELLS FROM PATHOPHYSIOLOGICAL LUNG BUT NOT BLOOD T CELLS

Derrick J. Gibbings, Yokanath Sekar, Marcus C.Y. Ng, Neil M. Skjodt, Eric Wong, and A. Dean Befus

From the Pulmonary Research Group, Department of Medicine, University of Alberta
Edmonton, Alberta, Canada

Abbreviations used: TCR (T cell receptor), FcR (receptor immunoglobulin Fc), BAL (bronchoalveolar lavage), Mr (molecular mass), PBMC (peripheral blood mononuclear cells), pI (isoelectric point), FBS (fetal bovine serum), ConA (concanavalinA), PHA (phytohaemagglutinin), SDS (sodium dodecyl sulfate), ARDS (acute respiratory distress syndrome), LAT (Linker for Activation of T cells)

Address for Correspondence: A. Dean Befus, 550B Heritage Medical Research Centre, University of Alberta, Edmonton, Alberta, Canada T6G 2S2, Telephone: (780) 492-5014, Facsimile: (780) 492-5329, email: dean.befus@ualberta.ca

Keywords: CD8α / structure / associations / T cell / monocyte / bronchoalveolar lavage / human / MHC class I / F1 ATP synthase / ATPase

3.1. Introduction

CD8α is expressed by T cells, dendritic cells, and in certain species (human and rat, but not mouse) NK cells [1-3] monocytes (DG, submitted) [4;5] and macrophages [5-9]. CD8α binds MHC class I and on T cells CD8α enhances cytotoxic T cell responses mediated by TCR (reviewed in [10]). On monocytes CD8α appears to co-operatively enhance monocyte responses mediated through FcγR (DG, submitted). Monocyte TNF release and phosphorylation of linker for activation of T cells (LAT) instigated by immune complexes was amplified by co-stimulation through CD8α. Interestingly, the presence of human or rat CD8(+ve) monocytes/macrophages also associates with pathology of immune complex-mediated diseases in some of the following models: ischemia [11], glomerulonephritis [12], experimental allergic encephalomyelitis [13], allograft rejection [4], and dengue fever [14].

While several studies have previously noted that some anti-CD8α mAb bind selectively to T cells, thymocytes [15], macrophages [16], and potentially NK cells [17], none of these studies have pursued the reason behind binding of certain anti-CD8α mAb to some but not all CD8(+ve) cell types.

We find that anti-CD8α mAb D9 binds human monocytes and T cells from bronchoalveolar lavage (BAL) from 25% of pathological samples tested, but not blood T cells. Our data suggests many of the known changes in CD8, such as hetero vs. homodimerization and sialylation, do not affect binding of D9. While it is unclear why D9 binds monocytes but not T cells from blood, one remaining interpretation is a structural change in CD8α.

3.2. Materials and Methods

3.2.1. Antibodies

Isotype control antibodies were mouse IgG_1 and IgG_{2a} (Sigma, St. Louis, MO), IgG_{2a}-FITC, -PE, and –biotin were from Caltag (Burlingame, CA). Anti-CD8α mAb used were: D9 and 32-M4 (Santa Cruz, CA), LT8 (Serotec, Raleigh, NC), B9.11 (Beckman-Coulter Canada Inc., Mississauga, ON), and Nu-Ts/c (Nicherei Corp., Tokyo, Japan), and 51.1 (gift of Dr. D. Burshtyn, University of Alberta). Anti-CD8α mAb OKT8 was purified from hybridoma supernatant by protein G affinity chromatography. Anti-CD14-PE, anti-CD14-FITC. Anti-F1 ATP synthase β subunit mAb was obtained from Mitosciences (Eugene, OR).

3.2.2. Cell recovery and culture

The promonocytic cell line THP-1 was maintained in media as recommended by American Type Culture Collection (RPMI 1640 medium, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate 0.05 mM 2-mercaptoethanol, and 10% fetal bovine serum [FBS]).

Human blood was collected into heparinized tubes. Red blood cells were sedimented by addition of 7 mL 6% dextran (Sigma) in RPMI 1640 (0.5 h, room temperature). White blood cells and plasma were underlayed with Ficoll-Paque Plus (Amersham Biosciences, Oakville, ON, Canada) and centrifuged (room temperature, 25 min, 200 g) without brake. The interface layer was removed and washed three times in PBS to obtain peripheral blood mononuclear cells (PBMC).

Lymphocytes and monocytes were separated from PBMC in some experiments. A CD14 positive selection kit and a handheld magnetic system (StemCell, Vancouver, BC) were used to enrich monocytes. Lymphocytes were enriched by depletion of cells adherent to tissue culture flasks (1 h, 37 C).

Human bronchoalveolar lavage (BAL) cells were collected at the University of Alberta Hospital by Drs. Wong and Skjodt with standard institutional consent allowing research use of samples from outpatients (12) or intensive care unit patients (5) undergoing diagnostic bronchoscopy. Patients with known diagnoses of HIV, HCV, or infection with organisms requiring containment above level 2 facilities were excluded. BAL samples were taken from radiographically identified disease lung subsegments. The flexible fibreoptic bronchoscope was wedged into the target subsegment with instillation of sterile preservative free saline (Baxter) in two or more 30 ml aliquots. With the injection of each aliqout bronchial blanching confirmed proper technique. A small portion of each clinical sample was aseptically split for immediate processing. Human thymus was a kind gift of Dr. John Elliott (University of Alberta). Thymi were obtained from discarded material following cardiac surgery. Ethics approval for all human material was granted by the University of Alberta/Capital Health Research Ethics Board.

3.2.3. Flowcytometry

Cells were kept on ice (4 C) throughout experiments. Non-specific mAb binding to cells was minimized with 5% milk, 0.1% bovine serum albumin (BSA) in PBS or with human Ig (50μg/mL, Bethyl Laboratories Inc., Montgomery, TX). Concentrations used were: anti-CD8α mAb (10 μg/mL), anti-mouse Ig-FITC Ab (1/100, STAR70, Serotec), anti-mouse Igtricolor (1/30, Caltag), and anti-CD14-FITC (1/50), Caltag). CellQuest (BD Biosciences) and WinMDI software was used to analyse data.

3.2.4. *T cell proliferation and neuraminidase treatment*

PBMC partially depleted of monocytes by adherence to plastic (1 h, 37 C) were stimulated for 20 h with phytohaemagglutinin (PHA), concanavalin A (ConA), CD3, or CD3 and CD28 (0.1-10 μg/mL). Anti-CD3 (OKT3, 10 μg/mL) and anti-CD28 (BD Biosciences, 2 μg/mL) mAb were attached to 96 well plate wells by incubation for 2 h at room temperature. In some experiments PBMC (10^7/mL) were treated or untreated with 100 mU/mL of *Clostridium perfringens* neuraminidase (Sigma) in PBS (pH 7.0) with 1 mM $CaCl_2$ for 1 h (37 C, 5% CO_2). Removal of sialic acid was confirmed by comparing untreated and treated cells for aggregation induced by the sialic acid specific lectin from *Sambucus nigra* (Calbiochem, San Diego, CA). Thereafter cells were kept on ice in the presence of sodium azide (0.02%) and reactivity of anti-CD8α mAb was analyzed by flow cytometry.

3.2.5. *Monoclonal antibody affinity chromatography*

OKT8 mAb at 5-10 mg/mL in 0.1 M HEPES pH 7.5 was coupled to pre-washed N-hydroxysuccinimidyl-activated agarose beads (Sigma) at 4 C for 1 h. Remaining active sites were blocked by incubating with 0.1 mL 1 M ethanolamine pH 8 at 4 C for 1 h. Human thymus was dissociated on metal mesh in cold PBS and passed through a 40 μM sieve (Fisher Scientific Ltd., Nepean, ON, Canada). Thymic cells were lysed with 1% triton X-100 in PBS with complete mini anti-protease cocktail tablets (Roche Applied Science, Laval, PQ, Canada). Supernatant remaining after 1000 g, 12,000 g, and 100,000 g centrifugations was loaded on columns. Columns were washed with 30 volumes lysis buffer, 20 volumes 10 mM Tris 0.5% triton X-100 300 mM NaCl pH 8, 20 volumes 10 mM sodium phosphate 0.5% triton X-100 450 mM NaCl pH 10, and eluted with 0.05 M diethylamine 0.5% triton X-100 650 mM NaCl pH 11.5. Fractions of 1.5 mL were collected into 50 μL 1 M Tris HCl pH 6.7.

3.2.6. 2-D electrophoresis

THP-1 whole cell lysate was prepared in 2% triton X-100, 2% CHAPS, 10% isopropanol, 12.5% isobutanol, 5% glycerol, 5 M urea, 2 M thiourea, 1% tributyl phosphate (Bio-Rad Laboratories, Hercules, CA), and 2% carrier pI 3-10 ampholytes (Bio-Rad). Lymphocyte and monocyte lysates were prepared using the 2-D cleanup kit (Bio-Rad) and resuspended in IPG strip rehydration buffer (Bio-Rad) with 2% carrier pI 3-10 ampholytes (Bio-Rad) for analysis. Affinity chromatography fractions were precipitated in 10% TCA (30 min, 4 C), centrifuged (12 000 g, 5 min), and washed 2 times in acetone (-20 C), before resuspension in rehydration buffer (Bio-Rad) for 2-D electrophoresis. Lysates were focused on 7 cm pI 3-10 strips (Bio-Rad), at voltage maximums of 50 V 10 min, 250 V 30 min, 750 V 1 h, and 8 000 V for 13 000 V h. Strips were equilibrated, reduced, and treated with iodoacetamide according to supplier directions (Bio-Rad) before running second dimension.

3.2.7. MALDI-QTOF

Bands were excised and an automated in-gel tryptic digestion was performed on a Mass Prep Station (Water, USA). The gel pieces were de-stained, reduced (DTT), alkylated (Iodoacetamide), digested with trypsin (Promega Sequencing Grade Modified) and the resulting peptides extracted from the gel and analyzed via LC/MS/MS. LC/MS/MS was performed on a CapLC HPLC (Waters, USA) coupled with a Q-ToF-2 mass spectrometer (Waters, USA). Tryptic peptides were separated using a linear water/acetonitrile gradient (0.2% Formic acid) on a Picofrit reversed-phase capillary column, (5 micron BioBasic C18, 300 Angstrom pore size, 75 micron ID x 10 cm, 15 micron tip) (New Objectives, MA,USA), with an in-line PepMap column (C18, 300 micron ID x 5 mm), (LC Packings, CA, USA) used as a loading/desalting column. Protein identification from the generated MS/MS data was done searching the NCBI non-redundant database using Mascot Daemon (Matrix Science, UK). Search parameters included carbamidomethylation of cysteine, possible oxidation of methionine and one missed cleavage per peptide.

3.2.8. Purification of Mitochondria

Crude mitochondrial fractions were prepared by lysing THP-1 cells with 1% triton X-100 (Sigma) in PBS with Protease Arrest Cocktail (Genotech, St. Louis, MO) for 20 min (4 C). Lysate was centrifuged at 1000 g for 10 min. Retrieved supernatant was centrifuged at 12 000 g for 10 min. Pelleted crude mitochondrial fraction was washed once. Pure mitochondrial fractions were prepared as previously described [18]. Briefly, cells in 10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 7.5 were left for 5 min on ice and homogenized with a Potter-Elvehjem homogenizer. Sucrose was added to 250 mM using 2 M sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.6. Supernatant remaining after two centrifugations at 1300 g, 5 min was centrifuged at 15 000 g for 15 min. The mitochondrial pellet was washed three times with 250 mM sucrose buffer and resuspended. Centrifugation (70 000g, 40 min) on a discontinuous sucrose gradient (1-1.7 M) was used to obtain a highly enriched mitochondrial fraction from the interface. Mitochondria were washed twice in 250 mM sucrose buffer before use.

3.2.9. Immunoprecipitation and ELISA of F1 ATP synthase complex

Crude mitochondrial fractions from THP-1 were suspended in 0.18 mL 50 mM Tris-HCl pH 7.5 and 20 μL of n-dodecyl-β-D-maltoside (Calbiochem, San Diego, CA) was added. After 30 min on ice the suspension was centrifuged at 21 000 g for 30 min. Supernatant was incubated with 10 μL F1 ATP synthase immunocapture beads (Mitosciences, Eugene, OR) for 3 h at room temperature. Beads were washed three times with 50 mM Tris-HCl pH 7.5 0.05% n-dodecyl-β-D-maltoside, eluted with 100 μL 0.1 M glycine pH 2.5, 0.05% n-dodecyl-β-D-maltoside, and pH neutralized with 5 μL 1 M Tris pH 8.

Purified F1 ATP synthase was diluted (5 μL to 100 μL) in 0.1 M Na_2HPO_4 pH 8.9 to coat wells of 96 well plates (Falcon 353912) for 2 h at room temperature. Wells were blocked with 150 μL 2% FBS in PBS for 30 min at room temperature. All subsequent washes were performed with 2% FBS in PBS. Wells were washed twice, incubated with primary antibody (45 min, ice), washed three times, incubated with anti-mouse Ig-HRP (Pierce, Rockford, IL) and developed with TMB (Sigma). Reaction was stopped with 2 N H_2SO_4 and read at 490 nM on a PowerWave XS Reader (Bio-Tek, Winooski, VT) using KC4 software (Fisher Scientific, Nepean, ON).

3.2.10. Purification of Cell Surface Proteins

Isolated cells had their surface proteins labeled with biotin using 0.5 mg/mL EZ-Link Sulfo-NHS-Biotin (Pierce) in PBS pH 7.9 for 30 min. After washing three times to remove excess biotin, cells were lysed in 1% triton X-100 with Protease Arrest Cocktail (Genotech). Nuclear material was removed by centrifugation at 1000 g, and biotinylated proteins were purified from supernatant using a monomeric avidin column (Pierce) according to supplier instructions. In brief, the 2 mL column was pre-cleaned with 12 mL pH 2.8 glycine buffer, rinsed with 12 mL PBS, and the sample was loaded. After washing column with 12 mL of PBS, biotinylated proteins were eluted with buffer containing 2 mM biotin.

3.3. Results

3.3.1. Anti-CD8α MAb D9 Does Not Bind Blood T cells Despite Binding Monocytes

Flow cytometry of peripheral blood mononuclear cells (PBMC) with seven anti-CD8α mAb was performed. As expected, between 15 and 30% of gated blood T cells (FSC/SSC and $CD3^{hi}$ gated, Fig. 1, top) and all monocytes (FSC/SSC and $CD14^{hi}$ gated, Fig. 1 bottom) bound six anti-CD8α mAb (OKT8 is shown as representative of 51.1, LT8, B9.11, LT8 and Nu-Ts/c). In contrast anti-CD8α mAb D9 did not detectably bind blood T cells, but did bind monocytes (Figure 3-1).

3.3.2. Anti-CD8α Clone D9 binds T cells Obtained by BronchoAlveolar Lavage from Some Patients

Anti-CD8α clone D9 binds CD8(+ve) blood monocytes and AM but not blood T cells, or a CTL clone (data not shown). We obtained samples from 17 consecutive patients to present a spectrum of lung diseases (Table 3-1), and determine whether D9 binds to BAL T cells in any circumstances. CD8α(+ve) lymphocytes were found in 12 of 17 samples (70%, Table 3-1). In three patients (25% of patients with CD8(+ve) cells) D9 bound a similar percentage of T cells, at a similar level (mean fluorescence intensity > 100) as other anti-CD8α mAb (Fig. 1B top, FSC/SSC gated). In two patients with acute respiratory distress syndrome (ARDS, 16.6% of patients with CD8(+ve) cells) D9 reacted at mid levels (mean fluorescence intensity < 100) with cells that could not be distinguished as monocytes or lymphocytes by FSC/SSC scatter (Fig. 1B bottom). Thus, D9(+ve) lymphocytes were found in 2/2 patients with suspected cancer and 1/6 patients with sarcoidosis, 0/2 patients with pneumonia, and 0/2 patients with non-sarcoid interstitial lung disease (Table 3-1).

3.3.3. Clone D9 is Not Specific for CD8αα Dimers or an Epitope Obscured by Sialic Acid

Monocytes express CD8αα and bind anti-CD8α clone D9 while most blood T cells express CD8αβ and do not bind D9. To test whether anti-CD8α clone D9 binds CD8αα but not CD8αβ dimers we induced CD8αα dimers on T cells by activating them with ConA or PHA for 5 days [19;19;20;20]. A portion of CD3+ T cells, likely CD8αβ+ T cells [19;20], expressed high levels of CD8α (Figure 3-1C, anti-CD8α mAb OKT8). Remaining cells expressed low levels of CD8α, suggesting they are CD4+ T cells with PHA-induced CD8αα homodimers as characterized by others [20](Fig 1C, anti-CD8α mAb OKT8) [19;20]. Anti-CD8α mAb OKT8, but not D9 bound to cells expressing low levels of CD8α (Figure 3-1C) suggesting D9 is not specific for CD8αα homodimers or CD8αβ heterodimers, as anti-CD8β clone 2ST8.5H7 is [21].

Sialylation of CD8α [22], like CD8β [23;24] changes after activation of mature or immature T cells [25]. However, binding of clone D9 to blood T cells was not obscured by sialylation, as its removal with neuraminidase had no affect on binding of clone D9 to blood T cells (Figure 3-1D).

3.3.4. In Denatured Form Clone D9 Binds CD8α from Blood T Cells

D9 does not bind CD8α in its native state on the surface of blood T cells. To test whether clone D9 binds CD8α on blood T cells in a denatured form we performed western blot. Surprisingly, D9 detected proteins at two Mr (32 and 52 kDa) in a variety of cell types (Figure

3-1E). The 52 kDa protein was detected in all cell types, including lung epithelial cells that served as a presumably CD8(-ve) control, suggesting D9 cross-reacted with a protein other than CD8. The lower Mr protein was 32 kDa in agreement with the Mr of CD8α (Figure 3-1G). Similar 32 kDa proteins were detected by western blot of thymus lysate, blood lymphocytes, immature monocytes (THP-1), mature *ex vivo* monocytes, and Mφ differentiated with GM-CSF from blood monocytes, but not in lung epithelial cells (A549, negative control).

We tested whether clone D9 recognized differentiable forms of CD8α when proteins were further separated by 2-D electrophoresis. Across three donors highly similar dots were observed for CD8α with anti-CD8α mAb D9 in monocytes and lymphocytes (Figure 3-1F is a representative example).

3.3.5. In Denatured Form Clone D9 Binds CD8α and the F1 ATP Synthase β Subunit

We set out to confirm that the 32 kDa protein bound by anti-CD8α clone D9 is CD8α and to determine the identity of the 52 kDa protein. Partially enriched preparations of human CD8α were separated by 2-D electrophoresis, and western blot with D9 was performed (Figure 3-1G). Identically prepared 2-D gels were silver stained and aligned with western blots to identify and extract spots detected by D9 for trypsin digestion and peptide sequencing. This demonstrated that the 32 kDa protein recognized by anti-CD8α clone D9 is CD8α, and suggested D9 recognized the F1 ATP synthase β subunit at 52 kDa (Figure 3-1G).

The F1 ATP synthase, including the β subunit synthesizes and breaks down ATP at the inner mitochondrial membrane. In addition to a heart mitochondrial preparation from commercial sources, mitochondria were purified from THP-1 monocytic cells which express CD8α, using a method that excludes endoplasmic reticulum, a contaminant of crude mitochondrial preparations, and immature CD8α [18]. Western blot with D9 detected only a 52 kDa protein in purified mitochondria, consistent with recognition of F1 ATP synthase β subunit (Figure 3-2A).

The F1 ATP synthase complex was immunoprecipitated from a crude mitochondrial preparation using anti-F1 ATP synthase antibody and analyzed by ELISA. Of a panel of anti-CD8α mAb only D9 bound immunoprecipitated F1 ATP synthase (Fig. 2B), confirming the immunoprecipitated material was not contaminated with CD8α, and that D9 binds the F1 ATP synthase. The F1 ATP synthase was detected in all immunoprecipitations at levels slightly above background by anti-F1 ATP synthase β subunit mAb, confirming the successful immunoprecipitation of the F1 ATP synthase.

Analysis with standard sequence or structural alignment programs like ClustalW or VAST detected no extended regions of similarity between CD8α and the F1 ATP synthase β subunit (not shown). Thus, the epitope recognized by D9 in the denatured F1 ATP synthase β subunit is likely an isolated motif with minimal resemblance to CD8α.

3.3.6. F1 ATP synthase β subunit is not found on the surface of monocytes

While located in the mitochondria of most cell types, F1 ATP synthase with intact enzymatic function, is also found on the surface of endothelial cells [26;27]. As anti-CD8α mAb D9 binds the F1 ATP synthase β subunit as well as CD8α, the selective localization of F1 ATP synthase on the surface of monocytes may account for D9 reactivity with these cells. D9 recognized only a 32 kDa protein (CD8α) among purified cell surface proteins of monocytes and T cells (Fig. 2C). However, the 52 kDa F1 ATP synthase β subunit could still be present at low levels. Thus, we tested whether the F1 ATP synthase was detected at the surface of monocytes by flow cytometry. In this system D9 binds monocytes (Fig. 1A). The anti-F1 ATP synthase β subunit mAb we used, has been used by others to detect the F1 ATP synthase β subunit at the surface of endothelial cells [26;27]. However, we were unable to detect F1 ATP synthase β subunit at the surface of monocytes with this mAb (Fig. 2D), using the same conditions in which D9 binds monocytes (Figure 3-1B).

3.4. Discussion

We demonstrate that anti-CD8α mAb D9 binds CD8α on the surface of monocytes but not T cells from blood. This difference may be worthy of further study for reasons presented later in the discussion.

D9 bound at equivalent levels to monocytes or BAL T cells as other anti-CD8α mAb or not at all, and detected a 32 kDa form of CD8α from the monocyte surface, and inhibited binding of MHC class I tetramers to monocytes (DG, submitted). Therefore D9 likely binds CD8α, not the F1 ATP synthase in a native form at the surface of monocytes. Thus the cross-reactivity of anti-CD8α mAb D9 should not materially affect our previous conclusions regarding CD8α on monocytes (DG submitted). That D9 binds the F1 ATP synthase β subunit at the surface of D9+ T cells from lung lavage remains a possibility.

The β subunit of the F1 ATP synthase was present with CD8α after enrichment by immunoaffinity with the anti-CD8α mAb OKT8. OKT8 did not bind immunoprecipitated F1 ATP synthase directly, suggesting that the F1 ATP synthase was present after OKT8 immunoaffinity chromatography as a non-specific contaminant, or possibly a co-precipitated partner.

D9 detected limited variation of CD8α in the range of pI 6-7 (Figure 3-1F, G). No unique form of CD8α was detected by D9 on monocytes compared to T cells to account for the selective binding of D9 to monocytes. Therefore, if a modification of CD8α itself on T cells prevents the binding of D9, this modification must be of minimal charge or Mr.

Our other data (DG, submitted), which resembles that of others [28;29] suggests a heterogeneous array of CD8α molecules from pI 6-9 by 2-D electrophoresis, in comparison to the limited variation of CD8α detected by D9 (pI 6-7, Figure 3-1F, G). This suggests D9 binds only some versions of CD8α, at least in denatured form. Others found a highly similar 2-D electrophoretic pattern for immunoprecipitated CD8α from thymocytes [30], as we found here with D9 on monocytes and lymphocytes. This suggests other anti-CD8α mAb may share the selective binding to certain cell types or forms of CD8α that clone D9 demonstrates. On the other hand, D9 appears to recognize the same quantity, and therefore probably the same heterogeneity of CD8α on the monocyte surface, in native form, as other anti-CD8α mAb (Fig. 1A, and [DG, submitted].

Structural variants of CD8α are one possible explanation for D9 binding to monocytes but not lymphocytes. Two structural variants of CD8α were observed in a crystal structure of CD8αα binding to MHC class I [31]. Indeed, other evidence suggests that some anti-CD8α mAb may cause a structural change that causes CD8 to have a higher binding affinity for MHC class I [32-34] and a heightened ability to activate T cells [33]. One possible structural change in CD8α is evident. CD8α contains three cysteines in a triangulation that suggests a small conformational change may allow the classical B-F strand disulfide bond of Ig domains to switch to a B-C strand disulfide bond. Such changes occur in other Ig-domain containing proteins like CD4, and are regulated by the release of specific thiol redox proteins [35].

D9 bound at equivalent levels to monocytes or BAL T cells as other anti-CD8α mAb or not at all (blood T cells). Only a small fraction [36-38] of CD8 associates on the same cell with[39] CD3δ [36], TCR [38], or the small extracellular domain of LAT [40;40], suggesting that these inter-molecular associations would not account for D9 binding to monocytes but not T cells[36;37]. Furthermore, D9 did not bind T cells fixed 10 minutes after activation through CD3/CD28 or PHA (data not shown), suggesting that D9 binding to CD8α was not affected by inter-molecular associations occurring during T cell activation.

Anti-CD8α mAb D9 inhibits binding of MHC class I tetramers to monocytes to a greater extent than two other anti-CD8α mAb (DG, submitted), thus the D9 epitope on CD8α may be close to MHC class I contact sites. Binding of CD8α on human blood T cells to MHC class I is not believed to be detectable in the absence of TCR, as in mouse T cells [23;24;47]; allowing their use for detection of antigen-specific T cells. In contrast CD8α on monocytes bound MHC class I tetramers in the absence of TCR (DG, submitted), suggesting CD8α on these cells has a higher affinity or avidity for MHC class I. Differences in monocyte CD8α detected by D9 may allow it to bind MHC class I tetramers in the absence of TCR. Several studies have previously noted that some anti-CD8α mAb bind selectively to T cells, thymocytes [15], macrophages [16], and potentially NK cells [17], and separate work has shown that some of these anti-CD8α mAb bind regions of CD8 involved in MHC class I binding [1], like D9.

CD8α can associate on the same cell, or *in cis*, with CD81 [41;42]) and MHC class I [43-45]. Others have shown that Ly49A receptors bind *in cis* to MHC class I, thereby preventing NK cell activation by Ly49A binding to MHC class I molecules on a target cell, or *in trans* [46]. This *in cis* binding of Ly49A to MHC class I obscures binding of some mAb to Ly49A [46]. As D9 appears to bind an epitope on CD8α close to its MHC class I binding site (above), an *in cis* association of MHC class I and CD8α is a particularly attractive explanation for binding of D9 to monocytes but not T cells.

Our data suggests that CD8α on monocytes can be differentiated from CD8α on blood T cells by anti-CD8α mAb D9. These differences may be associated with changes in the ability of CD8 to bind MHC class I and activate monocytes and T cells.

3.5. Figure Legends

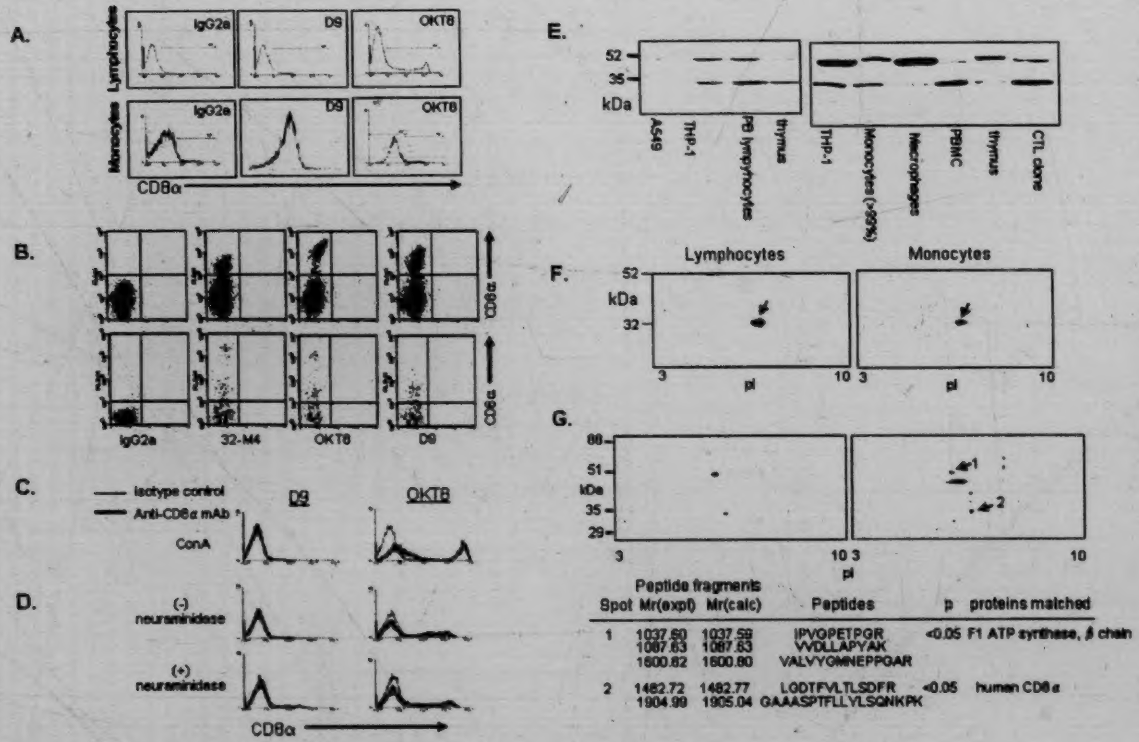
Figure 3-1. Anti-CD8α mAb D9 binds monocytes but not T cells from blood.

(A) Anti-CD8α mAb D9 does not bind to blood T cells (gated as CD3^{hi}), although CD8α is detected by other anti-CD8α mAb (OKT8). D9 binds blood monocytes (gated as CD14^{hi}) similarly to other anti-CD8α mAb (OKT8). In (A) IgG2a demonstrates binding of isotype control mAb and OKT8 is shown as representative of anti-CD8α mAb 51.1, B9.11, LT8, Nu-Ts/c, and 32-M4. Results are one representative of at least five experiments. (B) Dotplots with anti-CD8α mAb 32-M4 and OKT8 are shown as positive controls, and IgG2a as a negative control for binding of anti-CD8α mAb D9 to cells retrieved by BAL. Top, in three of 12 patients D9 bound lymphocytes (gated by characteristic FSC/SSC scatter) at levels equivalent to other anti-CD8α mAb. Results shown are from a patient with lingual cancer. Bottom, in two of 17 patients with ARDS D9 bound low levels of BAL cells that could not be separated as monocytes and lymphocytes by FSC/SSC scatter. (C) D9 binding is not dependent on expression of CD8αα homodimers. CD8αα homodimers were induced on T cells by activation of lymphocytes for 5-8 days with PHA or ConA. Results are representative of three experiments. (D) Removal of sialic acid moieties does not affect binding of D9 to lymphocytes compared to isotype control (IgG2a). Lymphocytes were treated as above, then kept on ice with sodium azide to prevent renewal of cell surface CD8α. Binding of anti-CD8α mAb OKT8 is included as a control for detection of CD8α. Results are representative of three experiments. (E) Human CD8α is expected to be 32 kDa. Anti-human CD8α mAb D9 detects proteins of 32 and 52 kDa in T cells from several sources, monocytes and macrophages. (F) Proteins of similar pI were detected by D9 on monocytes and lymphocytes after two-dimensional electrophoresis. Spots assumed to be CD8α are indicated by arrows. (G) CD8α was immunoaffinity-purified from human thymus. CD8α enriched material was analyzed by two-dimensional electrophoresis followed by silver staining (right) and western blot with anti-CD8α mAb D9 (left). Alignment of silver stained gels with western blots demonstrated D9 bound proteins indicated by arrows 1 and 2. Proteins indicated by arrows 1 and 2 were sequenced after trypsin digestion by MALDI-QTOF. Calculated and theoretical Mr, peptide sequences determined, and probability of correct identification of protein is supplied.

Figure 3-2. Anti-CD8α mAb D9 binds the F1 ATP synthase, but the F1 ATP synthase is not present on the surface of blood monocytes.

(A) Anti-CD8α mAb D9 detects a mitochondrial 52 kDa protein, consistent with the F1 ATP synthase β subunit in two sources of purified mitochondria. Whole THP-1 (monocyte cell line) lysate is included as a control for D9 binding 32 and 52 kDa proteins. (B) Anti-CD8α mAb D9 binds F1 ATP synthase immunoprecipitated from crude mitochondria from THP-1. Other anti-CD8α mAb do not bind immunoprecipitated F1 ATP synthase, suggesting D9 specifically and uniquely binds the F1 ATP synthase. (C) Only the 32 kDa protein (CD8α) bound by D9 is localized to the surface of lymphocytes and monocytes. Whole THP-1 cell lysate is included to demonstrate position of 32 and 52 kDa bands detected by D9. Surface proteins were purified from lymphocytes and monocytes by biotinylation with membrane-impermeable reagents and avidin chromatography of cell lysate. (D) F1 ATP synthase is not detected at the surface of monocytes by flow cytometry. Anti-F1 ATP synthase β subunit mAb has been used by others to detect F1 ATP synthase at the surface of cells [27], but does not bind the surface of monocytes.

3.6. Figures



Spot	Peptide fragments Mr(expt)	Mr(calc)	Peptides	p	proteins matched
1	1037.60 1087.63 1600.82	1037.59 1087.63 1600.80	IPVGPETPGR VVDLLAPYAK VALVYGQMNEPPGAR	<0.05	F1 ATP synthase, β chain
2	1482.72 1904.99	1482.77 1905.04	LGDTFVLTLSDFR GAAASPTFLLYLSQNKPK	<0.05	human CD8α

Figure 3-1. Anti-CD8α mAb D9 binds monocytes but not T cells from blood.

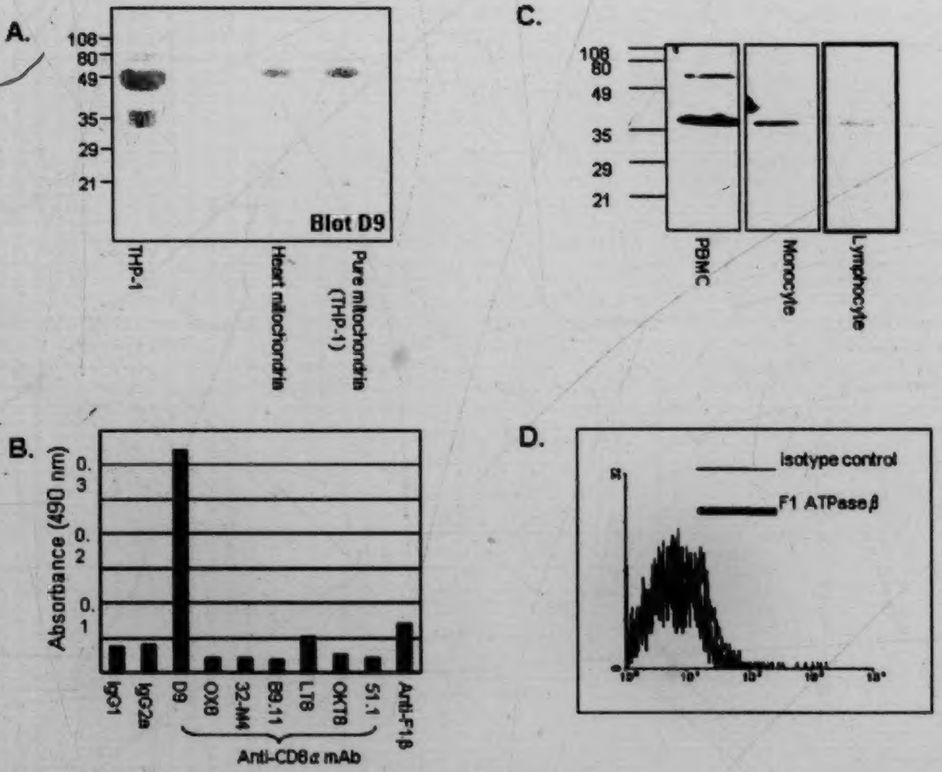


Figure 3-2. Anti-CD8α mAb D9 binds the F1 ATP synthase, but The F1 ATP synthase is not present on the surface of blood monocytes.

3.7. Table

Preliminary Diagnosis	D9 reactivity
Sarcoidosis	1/6
Non-Sarcoid intersitial lung disease	0/2
Cancer	2/2
ARDS	2/3 (weak)
Pneumonia	0/2

D9 high	25%
D9 weak	17%
D9+ of total	42%

Table 3-1. Anti-CD8α mAb binds CD8+ cells from bronchoalveolar lavage of some patients

3.8. Literature Cited

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4. MACROPHAGE MIGRATION INHIBITORY FACTOR HAS A MHC CLASS I-LIKE MOTIF AND FUNCTION

Derrick J. Gibbings‡, Alexandru F. Ghetu§, and A. Dean Befus‡

From the ‡Department of Medicine, 550 Heritage Medical Research Centre, University of Alberta, Edmonton, Alberta, Canada T6G 2S2 and §Ontario Cancer Institute, 610 University Avenue, Toronto, Ontario, M5G 2M9, Canada

Running title: MIF similarity to MHC class I

Address correspondence to: A. Dean Befus, Department of Medicine, 550 Heritage Medical Research Centre, University of Alberta, Edmonton, Alberta, Canada, T6G 2S2, Telephone: (780) 432-5032, Fax: (780) 492-5329, email: dean.befus@ualberta.ca

Keywords: NK cell / PIRB / CD74 / cross-reactive / cytotoxicity / MIF / macrophage migration inhibitory factor

4.1. Introduction

MIF is a secreted trimeric protein produced by immune cells, as well as by endocrine tissues (anterior pituitary gland, adrenal gland and hypothalamus) (reviewed [1]). MIF may modulate the immune response to enhance inflammation while reducing cell-mediated cytotoxicity. It is released in stress responses, such as after exposure to LPS and potentiates the effects of endotoxemia [2]. Several hormones released as part of the stress response, like MIF, regulate NK cell killing [3;4]. MIF is found in the immune-privileged uterine [5;6] and ocular environments where it respectively associates with lower rates of recurrent miscarriage [7] and inhibits LAK cell cytotoxicity [8;9]. Addition of MIF immediately prior to 4 h LAK cell cytotoxicity experiments inhibits LAK cell killing of uveal tumor and other target cells (e.g. YAC-1, RMA/S) [8;9]. In contrast, MIF has no effect on antigen-specific or allogeneic CTL cytotoxicity in *in vitro* experiments [9;10], suggesting MIF may act through a NK cell specific mechanism. MIF is also over-expressed in many tumors, suggesting that it may be involved in tumor growth, and/or provide a mechanism to escape NK cytotoxicity [8;11].

It has been proposed that MIF functions through more than one cell surface receptor [12-16]. Whether through a receptor or not, MIF can be internalized, and influence protein degradation, cell cycle, and transcription through binding c-Jun activation domain binding protein-1 (JAB1) a subunit of the COP9 signalosome [17;18]. However, MIF can also act as a thiol oxidoreductase in the presence of glutathione and a select group of other thiol containing compounds [19;20]. MIF also has isomerase activity towards non-physiological substrates [1].

While evidence suggests addition of reduced glutathione or a thiol-reducing environment increases NK cell lytic activity after several days [21;22], there is no evidence suggesting a short term effect of glutathione on NK cell activity. Moreover, MIF inhibits rather than increases NK cell lytic activity [8;9], suggesting MIF does not affect NK cell lytic activity through its thiol redox activity.

Classically MHC class I is composed of three distinct domains, including $\alpha 1$ and $\alpha 2$ that present peptide to TCR, $\alpha 3$ which binds to CD8, and the non-covalently attached $\beta 2$-microglobulin ($\beta 2$m) subunit. A growing family of MHC class Ib and MHC class I-like proteins, and mimics of MHC class I are being discovered in cancerous [23], stressed, and virally infected cells, as well as in viruses themselves [24;25]. Several of these MHC class I homologs lack structural parts of MHC class I, such as $\beta 2$m (e.g. MICA [26]) and/or the $\alpha 3$ region (e.g. Rae-1 [27]). Similarly, an increasing number of Ig and C-lectin family receptors on NK cells, T cells and monocytes contact exposed loops of MHC class I or its homologs (reviewed in [28]), suggesting that small regions of similarity to MHC class I may suffice to modulate immune responses instigated by MHC class I receptors.

We demonstrate that one of several monoclonal antibodies that recognize classical and non-classical rat MHC class I (clone OX18) also binds MIF. The epitope of the anti-MHC class I mAb OX18 was mapped on MHC class I, and identified an amino acid motif, PRPEG in MHC class I that is found with a three amino acid intervening sequence in MIF. OX18 bound a peptide synthesized to mimic this region of MIF. MIF partially inhibited binding of H2D^b tetramers of MHC class I to LAK cells, suggesting that MIF binds an LAK cell receptor through the conserved PRPEG motif. This may explain the effect of MIF on LAK cell activity, and provide an example of the complexity to expect from receptors for the extended MHC class I family proteins.

4.2. Materials and Methods

4.2.1. Antibodies and other reagents

Anti-rat MHC class I mAb OX18 and F16-4-4 were purchased from Serotec (Raleigh, USA). Anti-human MHC class I mAb W6/32 was from BD Biosciences (Missisauga, Canada). H137 anti-mouse and rat MHC class I polyclonal antibody was a gift of Dr. Kevin Kane (University of Alberta). Anti-mouse and rabbit Ig-HRP were from Pierce. NHS-activated agarose beads were purchased from Sigma-Aldrich (Oakville, Canada). Mouse recombinant MIF was a kind gift of Dr. Christine Metz (Picower Institute, NY, USA). H2-D^b tetramerized with Extravidin-R-Phycoerythrin (Sigma-Aldrich, St. Louis, USA) and loaded with influenza peptide ASNENMETM and produced by the CanVac tetramer core facility was a gift of Dr. Kevin Kane, University of Alberta. Immunoaffinity purified $H2K^b$ was also a gift of Dr. Kevin Kane.

4.2.2. MALDI-QTOF Peptide Sequencing

Bands were excised and an automated in-gel tryptic digestion was performed on a Mass Prep Station (Water, USA). The gel pieces were de-stained, reduced (DTT), alkylated (Iodoacetamide), digested with trypsin (Promega Sequencing Grade Modified) and the resulting peptides extracted from the gel and analyzed via LC/MS/MS. LC/MS/MS was performed on a CapLC HPLC (Waters, USA) coupled with a Q-ToF-2 mass spectrometer (Waters, USA). Tryptic peptides were separated using a linear water/acetonitrile gradient (0.2% Formic acid) on a Picofrit reversed-phase capillary column, (5 micron BioBasic C18, 300 Angstrom pore size, 75 micron ID x 10 cm, 15 micron tip) (New Objectives, MA, USA), with an in-line PepMap column (C18, 300 micron ID x 5 mm), (LC Packings, CA, USA) used as a loading/desalting column. Protein identification from the generated MS/MS data was done searching the NCBI non-redundant database using Mascot Daemon (Matrix Science, UK). Search parameters included carbamidomethylation of cysteine, possible oxidation of methionine and one missed cleavage per peptide.

4.2.3. Monoclonal antibody affinity chromatography

OX18 (anti-rat MHC class I) or OX21 (isotype control mouse IgG1) at 5-10 mg/mL in 0.1 M HEPES pH 7.5 was coupled to pre-washed N-hydroxysuccinimidyl-activated agarose beads (Sigma) at 4 C for 1 h. Remaining active sites were blocked by incubating in 0.1 mL 1 M ethanolamine pH 8 at 4 C for 1 h. Rat cultured mast cell line (RCMC) was lysed with 1% triton X-100 in PBS with Complete Mini anti-protease cocktail tablets (Roche Applied Science, Laval, PQ, Canada). Supernatant remaining after 1000 g, 12,000 g, and 100,000 g centrifugations was loaded on columns. Columns were sequentially washed with 30 volumes lysis buffer, 15 volumes 10 mM Tris 0.5% triton X-100 pH 8, 15 volumes 10 mM Tris 0.5% triton X-100 pH 8.2 150 mM NaCl, 15 volumes 10 mM sodium phosphate 0.5% triton X-100 150 mM NaCl pH 10, 15 volumes 10 mM sodium phosphate 0.5% triton X-100 300 mM NaCl pH 10 and eluted with 0.05 M diethylamine 0.5% triton X-100 650 mM NaCl pH 11.5. One and a half mL fractions were collected into 50 µL 1 M Tris HCl pH 6.7.

4.2.4. Enzyme-linked Immunosorbent Assays (ELISA)

One hundred µL of 1/20 dilutions in PBS of MHC class I preparations or 0.02 µg/mL MIF in PBS was incubated in 96 well plates (polyvinyl chloride flexible plates, BD Biosciences [Missisauga, Canada]) for 2 h at room temperature. Wells were emptied and filled with 150 μL 2% FBS in PBS for 0.5 h. After three washes in 2% FBS primary antibodies (OX18, F16-4-4, etc.) were added in 100 μL (10 μg/mL) and incubated 45 min on ice. After another three washes in 2% FBS, anti-mouse or anti-rabbit Ig-HRP was added at 1/500 dilution and incubated 45 min on ice. After six washes in 2% FBS wells were developed with o-Phenylenediamine dihydrochloride (OPD) from Sigma. Development was stopped with 2N H_2SO_4. Color was read at 490 nm on a Bio-Tek (Winooski, VT) PowerWave XS spectrophotometer using the KC4 version 3.3 program.

MIF peptide and a scrambled version of the same peptide with charged amino acids mutated to similarly charged but different amino acids were synthesized by the Alberta Peptide Institute. MIF-(9-18) Ac-NVPRASVPEG-$CONH_2$, and scrambled peptide, Ac-SAGVDNAGKA-$CONH_2$ were synthesized using an Applied Biosystems Model 430A Peptide synthesizer and purified by HPLC to >95% purity. For ELISA of MIF peptide, peptide (100 μg/mL, 100 μL) was coated on Nunc Maxisorp 96 well plates in 0.1 M Na_2HPO_4 pH 8.85 for 2 h at room temperature. Wells were blocked with 10% FBS in PBS (150 μL, 1 h), and washed three times with PBS before incubation with primary antibody (5 μg/mL, 20 min, 4 C). After five washes with ice-cold PBS, anti-mouse Ig-HRP (Pierce [Rockford, IL, USA], 1/200) was added (20 min, 4 C). After six washes with PBS with 0.1% Tween-20 ELISA was developed with TMB substrate (BD Biosciences), stopped and analyzed at 450 nm as above.

4.2.5. Silver stain and western blot analysis

Proteins were diluted in denaturing SDS-PAGE sample buffer (Bio-Rad, Hercules, CA) with 2% 2-mercaptoethanol (Sigma) and placed in boiling water for 5 min. Proteins were run on 4-15% pre-cast gradient gels (Bio-Rad) and silver-stained with the PlusOne Silver Stain kit (Amersham Biosciences, Piscataway, USA) without glutaraldehyde, or transferred to PVDF membrane (Millipore). For western blot antibodies were diluted 1/1000 (H137, OX18, anti-MIF) or 1/10000 (anti-mouse or rabbit-HRP) in 5% milk in Tris-buffered saline, pH 8.2. Western blot was developed with Femto-Sensitive reagents from Pierce.

4.2.6. Binding of MHC class I tetramers to LAK cells

Splenocytes were harvested from 8-12 wk male Balb/c mice (Health Sciences Lab Animal Services in-house breeding colony, University of Alberta). Red blood cells were lysed for 5 min in ammonium chloride buffer. After 3 washes cells were cultured for 4 d with 1000 U/mL IL-2 (a gift of Dr. Kevin Kane, University of Alberta) as described [8]. After 4 d non-adherent and adherent cells (removed with 1 mM EDTA in PBS, 1 min) were harvested and washed three times in PBS with 0.02% NaN_3 at 4°C.

Cells were incubated (10^6 cells/well in 30 μL) for 20 min with 20 μg/mL recombinant MIF or 20 μg/mL BSA (Bio-Rad) in PBS with 0.02% NaN_3 at 4°C. H2-D^b tetramer (0.3 μg or 1.5 μg) and anti-CD3 mAb (10 μg/mL, CalTag, Burlingame, CA) were added without washing. Cells were incubated a further 20 min. After washing two times data was collected using a FACScan (BD Biosciences), and analyzed using WinMDI 2.8. Geometric mean fluorescences of tetramer binding and anti-CD3 mAb binding (a control for MIF inhibition of binding another protein to LAK cells) were obtained. For each sample a percentage inhibition of binding due to MIF was calculated: geometric mean of MIF treated cells - geometric mean of BSA treated cells / geometric mean of BSA cells x 100%. Percent inhibitions were averaged and a t-test was used to assess significance of results ($p < 0.05$).

4.3. Results

Immunoaffinity purification of both rat MHC class I and class Ib using mAb clone OX18 has been published [29;30]. According to these studies and expression cloning studies of rat MHC complex, OX18 binds nearly all rat MHC class I and Ib proteins [31-34]. We used similar immunoaffinity protocols with OX18 to purify a heterogenous population of rat MHC class I from rat cultured mast cell (RCMC) line. Eluted MHC class I proteins, under increasingly stringent washes, were detected with OX18 in ELISAs. A small amount of OX18-reactive material eluted with pH 8.2 150 mM NaCl buffer (Figure 4-1A, fractions 3-7). This may be non-specifically bound material or MHC class I variants that conserve the OX18 epitope. Much greater quantities of OX18-reactive proteins resisted elution at pH 10.5 300 mM NaCl, but eluted with pH 11.5, 650 mM NaCl (Figure 4-1A). Thus, washes were routinely performed in similar order and pH 11.5, 650 mM NaCl was used to elute OX18 immunoreactive material.

The purity of the MHC class I population eluted from the OX18 column was analyzed by silver staining (Figure 4-1B). We observed a set of bands covering the Mr range of 45-55 kDa, which is consistent with previous results showing that classical and non-classical rat MHC class I proteins cover a Mr range of 40-55 kDa [29]. However, proteins of Mr not consistent with MHC class I were also observed, including two bands of approximately 25 and 30 kDa. Similar bands were observed by others using OX18 affinity columns and their identity and reason for their presence is unresolved [34]. Western blot of purified fractions was performed to confirm the presence of rat MHC class I and to inquire whether the 25 and 30 kDa bands were degradation products of MHC class I. A polyclonal (Figure 4-1B) and a mAb (OX18, Figure 4-1B) recognizing MHC class I, detected proteins at the expected Mr (45-55 kDa) for

MHC class I. An additional protein of 12 kDa was detected with an anti-MHC class I polyclonal antibody. This band may be due to reactivity with the β2m subunit of MHC class I used to immunize animals. Alternatively, it could be a degradation product of MHC class I, or an MHC class I homolog of 12 kDa, such as Zn-alpha2-glycoprotein (ZAG).

Peptide sequencing by MALDI-QTOF was performed to confirm the presence of components of MHC class I (heavy chain and β2m) and to identify the 25 and 30 kDa proteins found in preparations of MHC class I. The dominant bands at 45-55 kDa were identified as RT1.Aμ (Figure 4-2A,B), the classical MHC class I allele expressed by the cell line utilized for purification of MHC class I (RCMC). The 25 and 30 kDa proteins were identified as peroxiredoxin IV and triose phosphate isomerase (TIM) respectively (Figure 4-2B). Surprisingly, while the 12 kDa band was anticipated to be β2m, MIF was identified by MALDI-QTOF (Figure 4-2A,B).

Heat shock protein (hsp) 70, hsp 90, and albumin were also identified in fractions with purified MHC class I (Figure 4-2B). These proteins are often contaminants of immunoaffinity columns [35] perhaps due to hydrophobic interactions with unfolded proteins or other relatively non-specific interactions. To our knowledge, MIF has not been noted as a contaminant of affinity columns. To confirm the presence of MIF we used polyclonal anti-MIF Ab in western blot and detected MIF at the expected Mr of 12 kDa (Figure 4-2C). β2m was also detected in enriched MHC class I (Figure 4-2C).

To test the possibility that OX18 used to purify MHC class proteins also binds directly to MIF, we performed an ELISA using OX18 with rat MHC class I and recombinant mouse MIF (differs from rat MIF by one amino acid, >99% conservation). OX18 recognizes an epitope conserved by many MHC class I and Ib proteins [31-34]. If there was more than one region with significant similarity between MIF and MHC class I, other mAb recognizing conserved epitopes of MHC class I may also recognize MIF. Using a small panel of mAb against conserved epitopes of human and rat MHC class I only OX18 significantly bound to MIF (Figure 4-3A). F16-4-4 binds most rat classical MHC class I alleles [33], and bound purified MHC class I, but did not bind MIF. W6/32 binds a conserved epitope of human MHC class I alleles, but did not bind rat MHC class I or MIF. H137, the polyclonal antibody that binds mouse and rat MHC class I, did not significantly bind MIF.

MIF was eluted from OX18 mAb columns with pH 11.5 650 mM NaCl after washes with pH 10.5, 300 mM NaCl suggesting a substantial affinity of OX18 for MIF. Confirming this, ELISA demonstrated OX18 bound directly to recombinant murine MIF (Figure 4-3A). However, to ensure MIF binds specifically to the antigen binding site of OX18, we substituted OX18 with an isotype mAb on immunoaffinity columns and performed the series of elutions shown in Figure 4-1A again (Figure 4-3B). Analysis of all elution fractions by ELISA using the OX18 mAb showed no reactivity, indicating that MIF does not bind the isotype mAb and that MIF binds regions of OX18 involved in its antigen-specific binding. In contrast, denaturation of MIF appears to abrogate binding of OX18, as a 12 kDa protein was not detected by western blot with OX18 (Figure 4-1B).

We predicted that the 45 kDa protein detected by the anti-MIF polyclonal antibody is MHC class I and we wanted to test the ability of the anti-MIF polyclonal antibody to bind purified mouse H2K^b. Denatured H2K^b was not detected in western blots with anti-MIF polyclonal antibody (Figure 4-3C). Likewise, denatured MIF was not detected in western blots with anti-MHC class I polyclonal antibody (Figure 4-3C). Despite this we were able to detect binding of the anti-MIF polyclonal antibody to native H2K^b in ELISA (Figure 4-3C, right), suggesting that the H2K^b epitope recognized by anti-MIF polyclonal antibody comprises discontinuous regions of primary sequence brought together by H2K^b folding.

Recently details of the epitope recognized by OX18 were published and suggest OX18 binds the MHC class I $\alpha 3$ region. Two alleles of rat MHC class Ib (RT1.L^1 and RT1.L^2) differentially bound OX18 [32]. Changes in three amino acids in the $\alpha 3$ domain differentiate these alleles and any one alone or all of the changes together result in loss of OX18 binding: 194 R/S, 207 G/D, and 243 W/L. Furthermore, work cited as a personal communication in the same paper (R. Kirsch , E. Joly, G.W. Butcher) a region near amino acid 193 of RT1 was found to be important for OX18 binding [32]. We analyzed RT1 allele sequences based on published information about their reactivity with OX18 (and lack thereof) [36-41] (Figure 4-4A). Two expression clones non-reactive with OX18 (RT1.U cc22, cc23) [36] have a unique 244 A/S mutation that is not shared by any OX18 reactive sequences. Binding of OX18 to RT1.Aμ and expression clone 11/3R is diminished compared to other rat MHC class I [36]. The reduced binding of OX18 to RT1.Aμ and clone 11/3R is likely due to structural changes generated by a combination of amino acid differences. Therefore amino acids P193-194 and potentially W243-A244 likely form part of the OX18 binding epitope on MHC class I.

The OX18 epitope is highly conserved in rat MHC class I sequences (Figure 4-4A, shown on crystal structure Figure 4-4C), suggesting an important role in protein function. Some contact residues for β2m are in the vicinity however evidence suggests the OX18 binding site on MHC class I overlaps with the binding site for monocyte and/or NK cell receptors. While OX18 has no effect on CTL killing (like MIF), suggesting that it does not bind the α1/α2 domains [42] (supported by epitope evidence above), addition of $F(ab)_2$ fragments of OX18 to target cells activates NK cell killing [43]. Similarly inhibition of MIF activates LAK cell killing [8;9]. Thus OX18 may mask a region of MHC class I that interacts with an inhibitory receptor on rat NK cells.

The AB loop of the α3 domain that appears to contain the OX18 epitope, is the main contact loop (amino acids 193-196, 198, 248) for the inhibitory receptor Leukocyte immunoglobulin-like receptor subfamily B member 1 (LILRB1) in human MHC class I [44] (contact residues shown in Figure 4-4A, mapped on structure Figure 4-4C). LILRB1 also contacts β2m [44]. Paired-immunoglobulin-like receptor-B (PIRB), a proposed mouse homolog of the LILRB family of proteins [45] binds MHC class I in a β2m-dependent manner [46] suggesting that PIRB and LILRB1 may bind similar regions of mouse and human MHC class I respectively.

LILRB1 binds the AB loop of the α3 domain in human MHC class I that OX18 binds in rat MHC class I (Figure 4-4A,C). Significant polymorphism is found in the LILRB1/AB loop of human MHC class I [44] and mouse MHC class I (Figure 4-4B). Several mouse classical and non-classical MHC class I proteins conserve P193-R194 and/or most of the LILRB1 contact residues (193-196,198; these residues in rat RT1A are PRPE-D, and substantially differ from similar residues in human HLA). The non-classical mouse MHC class I molecule TL resembles the AB/LILRB1 loop of rat MHC class I most closely. Because OX18 retains binding to H2K^b (Figure 4-3C) residues 195-197 are likely not critical for its binding, as these residues are mutated in H2K^b (RT1.A - PEG, H2K^b - SQV). Residues P193-R194 may therefore be more important for OX18 binding of MHC class I. There is little polymorphism in the mouse equivalents of rat residues W243-A244 where OX18 may also bind.

Because OX18 denotes a functional epitope of MHC class I and binds MIF and mouse MHC class I (H2K^b, Figure 4-3C), MIF may contain a functionally important MHC class I epitope. To try to determine the location of the shared epitope on MHC class I and MIF we compared the sequences and structures of the two proteins.

MIF and the MHC class I antigen presentation domain each contain two alpha-helices overlayed in approximately similar orientation on a β-sheet, formed in part through trimerization of MIF. Although the architecture of MIF and MHC class I is similar, there is no conservation in the order of secondary motifs within the protein sequences and ClustalW did not detect any extended regions of amino acid similarity, suggesting that MIF and MHC class I are not related.

However, there is a single occurrence of PR in mouse and rat MIF at amino acids 11-12, similar to the PR important for OX18 binding to rat MHC class I (Figure 4-5A). Immediately following the proposed OX18 epitope P193-R194 in MHC class I is the sequence PEG (195-197) that is also found three amino acids downstream of P11-R12 in MIF (Figure 4-5A). In the crystal structure of MIF the P11-R12 and PEG (15-18) sequences are spatially linear, with the intervening sequence forming a loop extending toward the main body of MIF (Figure 4-5B). Furthermore the loop containing PR---PEG (11-18) is located in a flexible region of MIF, suggesting it may dynamically adapt to facilitate interaction with ligands. There is no WA sequence in MIF to mimic residues 243-244 of MHC class I. Thus MIF determinants of OX18 binding may include amino acids 11-12 (PR) and 16-18 (PEG).

To test the hypothesis that OX18 binds directly to MIF by binding a region including PR---PEG (11-18) a peptide was synthesized including this region (9-18, NVPRASVPEG). A scrambled and conservatively mutated version of this peptide (SAGVDNAGKA) was used as a control. Isotype control antibody did not bind MIF-(9-18) in comparison to scrambled peptide (Figure 4-5C). In contrast OX18 bound MIF-(9-18) compared to control peptide (Figure 4-5C). This suggests OX18 binds to MIF in a manner at least partly dependent on the PR---PEG (9-18) sequence proposed. Binding to MIF is lost after MIF denaturation (western blot, Figure 4-1B), suggesting that some tertiary structure of MIF may be important for strong binding of OX18 to MIF.

If a region of MHC class I is similar to MIF, MIF may be able to compete for MHC class I functions requiring binding to the region of similarity. Interestingly, MIF can inhibit killing mediated by NK cells [8;9] but not T cells [10]. Accordingly, we tested whether MIF could inhibit binding of tetramers of MHC class I (H2D^b) to LAK cells as a source of NK cells. Among known receptors for H2D^b are Ly49A/C/H/O/V [47], CD8 [48], and likely PIRB [46]. H2D^b conserves P193-R194 and W243-A244 important for OX18 binding. H2D^b like H2K^b has a change of charge at position 196 (E/K and E/Q respectively) in comparison to

RT1.A. However OX18 retains binding to H2K^b suggesting 196 is not essential to OX18 binding and therefore MIF similarity. Due to the hypothesized competitive nature of MIF inhibition of MHC class I tetramer binding, and the imperfect conservation of the MIF analogous sequence in H2D^b we anticipated the effect of MIF would be small.

Mouse splenocytes were cultured for 4 days in IL-2 to produce LAK cells. LAK cells were used because their cytotoxicity is inhibited by MIF [9]. Experiments were performed at 4 C in the presence of NaN$_3$ because MIF internalization and subsequent intracellular functions are temperature and energy dependent [49]. These conditions and the performance of experiments in PBS after several washes minimized the presence of glutathione and other thiol-redox partners of MIF. Thus, functions of MIF observed should involve MIF interaction with the LAK cell surface, and/or MHC class I tetramers. LAK cells consisted of populations of CD3^{high} T cells and CD3$^{low/negative}$ cells (Figure 4-6A). H2D^b tetramers bound >2% of CD3$^{low/negative}$ cells (NK, NKT cells), and CD3^{high} cells (Figure 4-6A) in agreement with literature suggesting H2D^b binds a very small percentage of Balb/c splenocytes [50]. For this reason 2.5 x 10^5 - 1.5 x 10^6 cells were analyzed in each experiment. Two controls were used. Binding in the presence of MIF was compared to binding in the presence of BSA as a control protein. As a control within each well, H2D^b binding and CD3 mAb binding were compared in the presence of a physiological amount of MIF (20 μg/mL). Binding of anti-CD3 mAb to CD3^{high}/H2D$^{b(high)}$ or CD3$^{low/negative}$ cells was not inhibited by MIF compared to control protein (BSA, Figure 4-6B). MIF trended toward the inhibition of binding of 0.3 μg H2D^b tetramer to CD3$^{low/negative}$ cells, but this effect was not statistically significant (Figure 4-6C). However, MIF significantly inhibited binding of 1.5 μg H2D^b tetramers (11.14% inhibition, $p<0.05$) to

CD3$^{low/negative}$ cells in the same wells (Figure 4-6C). Thus MIF can inhibit binding of MHC class I to non-T cells potentially due to a small region of similarity with MHC class I that allows it to compete for binding to a LAK cell receptor for MHC class I.

4.4. Discussion

After fortuitously purifying MIF with an anti-MHC class I mAb, we demonstrated that MIF is bound by anti-MHC class I mAb OX18. In addition, anti-MIF polyclonal antibody binds MHC class I, while anti-MHC class I polyclonal antibody does not significantly bind MIF. This apparent contrast may be due to the shared epitope being more antigenic within the context of MIF. Through knowledge of the epitope of OX18 we discovered a small region of similarity between MHC class I and a flexible region of MIF. Finally, we demonstrated that MIF can inhibit binding of MHC class I tetramers to CD3$^{negative/low}$ cells, suggesting that MIF may inhibit LAK responses through a shared epitope with MHC class I.

We initially purified MIF with an anti-MHC class I mAb from a rat mast cell line (RCMC). Others have shown that mast cells produce MIF [51]. MIF secretion by mast cells may contribute to the role of mast cells in anti-bacterial defense and septic shock [52]. MIF secretion by mast cells may suggest that there is a novel method of NK cell regulation by mast cells.

OX18 detected rat MHC class I, but did not detect MIF in denatured form by western blot, suggesting that the OX18 epitope is partially dependent on tertiary structure of MIF but not MHC class I. It was unexpected that OX18 bound MIF-(9-18), because it did not bind denatured MIF by western blot. MIF tertiary structure may bring distant parts of the MIF amino acid sequence into spatial proximity of the PRASVPEG sequence to enhance OX18 binding. Alternatively, MIF tertiary structure may provide a particular conformation of the PRASVPEG sequence that allows enhanced binding of OX18. OX18 may stabilize one of many peptide conformations in solution, allowing binding of OX18 to MIF-(9-18).

Our evidence suggests OX18 may bind PRPEG or the same sequence with an intervening three amino acids, PRASVPEG. Other protein-protein interactions have been described where intervening sequences in the ligand do not affect specific protein-protein interaction. One well-characterized example is the bulge of peptide outside the peptide binding groove of MHC class I when peptide length between contact residues is long [53]. In the case of MIF, the intervening sequence bulges toward the body of MIF and should not interfere with interaction with potential receptors.

Several activities of MIF appear to be dependent on associated molecules like glutathione for its thiol redox functions [19]. While we cannot exclude that MIF inhibition of tetramer binding is due to its thiol redox activity, we have attempted to minimize this possibility by performing short-term experiments with precise functional outcomes at 4°C in NaN_3 in the absence of serum (to minimize MIF internalization [49] and the presence or secretion of thiol redox partners of MIF in our system [19]). Furthermore, given that MIF contains a motif similar to MHC class I, and has the function associated with this motif in MHC class I, it is reasonable to suggest the inhibition of MHC class I tetramer binding by MIF we observed is due to this similar motif in MIF and MHC class I, and not another cause.

A binding site on MIF for catalysis of non-physiological substrates has been defined [54]. The enzymatic site of MIF is proximal to the thiol redox site, and this may explain the enzymatic site's uncertain relation to MIF function. The enzymatic and thiol-redox sites are distant on MIF from the proposed site of similarity to MHC class I. Little conformational change in MIF occurs upon binding enzymatic substrates or inhibitors of enzymatic activity [54], thus the two sites of MIF seem unlikely to directly influence each other.

Anti-MIF polyclonal antibodies have also been used in many *in vivo* studies [55-58]. Caution should be taken in interpreting parts of these experiments as we have shown that anti-MIF polyclonal antibodies also bind MHC class I, suggesting that anti-MIF polyclonal antibodies may inhibit MHC class I interaction with inhibitory receptors on monocytes, NK cells, B and T cells and/or induce antibody dependent cytotoxicity, the latter potentially pertinent in models of arthritis [57] and diabetes [56]. Others demonstrated that the inhibition of LAK cell killing by MIF could be neutralized by addition of anti-MIF polyclonal antibody [8;9]. However, the authors demonstrated that MIF inhibition and anti-MIF neutralization of LAK cell cytotoxicity also occurred when MHC class I negative target cells were used. Thus the interaction of anti-MIF antibody with MHC class I cannot explain their results. Our demonstration that MIF can inhibit binding of MHC class I to CD3$^{low/negative}$ cells suggests MIF may instead inhibit LAK cell killing through a shared region of similarity with MHC class I. As OX18 and anti-MIF polyclonal antibodies also bind mouse MHC class I, we did not attempt to inhibit the effect of MIF with these antibodies, as they would bind H2D^{b} directly and render conclusions impossible.

Recently, CD74, was defined as a receptor for MIF [12]. CD74 is also known as the invariant chain (Ii) involved in peptide loading and trafficking of MHC class II. Intriguingly CD74 co-immunoprecipitates with folded CD1d [59]and MHC class I (human and mouse) [60-62], an interaction annulled by the addition of allele-specific MHC class I peptides [62]. CD74 also interacts with the MHC class I chaperone calnexin [63;64]. This and other evidence suggests CD74 function(s) may not be exclusively associated with MHC class II folding intracellularly. CD74 is not exclusively expressed by MHC class II+ cells, or only intracellularly. Two to five percent of CD74 is found on the surface of CD74+ cells, and CD74 knockout mice have phenotypes not easily explained by MHC class II deficiencies (discussed in [12]). It is possible that CD74 is found on the surface of NK cells where it can bind MHC class I. This binding may be inhibited by MIF. CD74 does not appear able to activate the signaling pathways MIF activates, and CD74 did not account for all MIF binding to cells[12]. Therefore, different MHC class I binding receptors on different cell types, may be primary receptors or co-receptors for MIF with CD74.

MIF inhibits LAK cell cytotoxicity [8;9]. In this situation, if MIF acts through similarity to MHC class I, it either induces signaling through an inhibitory receptor, or inhibits signaling through an activating receptor. However, MIF may bind several receptors for MHC class I, allowing a diversity of potential responses. Activating receptors binding regions of MHC class I that might also bind OX18 may include PIRA [46], activating homologs of LLIRB1 recently cloned from rat and mouse [65], and CD8α [48]. Inhibitory receptors for MHC class I, Ly49A and C, contact residues 223 and 243 of α3, and thus contact part of the proposed OX18 epitope (W243-A244 but not the AB loop) [66-68], suggesting that the binding site of an uncharacterized Ly49 family member may more substantially overlap with the OX18 binding site shared by MIF. LILRB1's close structural [69] and functional [70] homologue LILRB2 has been proposed to be related to mouse PIRB [71;72]. PIRB binds several classical mouse MHC alleles [46] and HLA-G [73]. Like LILRB1 and -2 [70] mouse PIRB probably binds the AB loop of α3 domain [46]. Like LILRB1/2, PIRB has significant polymorphism and probably binds a polymorphic region (such as the AB loop of the mouse α3 domain, Figure 4-

4B) of MHC class I [44;45] like LILRB1/2 [46]. Thus, if MIF binds PIRB it may have more effect on binding of MHC class I alleles other than H2D^b, particularly given the polymorphism and charge heterogeneity of the AB loop of the $\alpha3$ domain among various mouse MHC class I. Despite evidence that rat NK cells express PIRB [18], *ex vivo* mouse splenic NK cells do not express PIRB [74]. After 4 d culture with IL-2 NK cells may express PIRB. Cells larger than lymphocytes were excluded in gating of LAK cells, thus it is unlikely that MIF inhibited tetramer binding to PIRB+ macrophages among our CD3$^{low/negative}$ LAK cells.

MIF was first characterized for its ability to inhibit the migration of macrophages, and signals through src kinases [18;75;76]. It is therefore intriguing that PIRB, a potential receptor for MIF, downregulates chemotaxis through src kinases [52]. MIF binding to PIRB could account for the effect of MIF on macrophage migration and other responses.

The inhibition of MHC class I tetramer binding by MIF is small, while the effect of MIF on LAK cytotoxicity is more pronounced [8;9]. Due to the influence of avidity, trimeric MIF may more capably compete with monomeric MHC class I *in vivo*, than with tetrameric MHC class I in our assay. Given the polymorphism of mouse MHC class I in the region similar to MIF it is possible MIF may more profoundly block binding of receptors to MHC class I alleles other than H2D^b. Moreover, *in vivo* the role of MIF may not be to compete with MHC class I for binding receptors, but rather when MHC class I is absent or present at low levels, such as in immune-privileged environments, to induce signaling that will inhibit NK cell cytotoxicity. MIF is trimeric and thus may induce some clustering of its receptors. On the other hand, it is not necessary that MIF inhibit LAK cell cytotoxicity solely through direct effects on LAK cell receptors for MHC class I. MIF may use receptors for MHC class I only as a docking site for entry into LAK cells, and inhibit LAK cell cytotoxicity via subsequent short term effects on transcription and protein degradation through interactions with Jab1. In this regard, it is interesting to note that MIF inhibits activation of transcription by AP-1, and inhibits c-Jun N-terminal kinase (JNK) [17]. AP-1 activates transcription early after induction of NK cell cytotoxicity [53], and JNK is activated by signaling through some activating NK cell receptors [54;77].

Interestingly, MIF has characteristics of many inhibitors of NK cell cytotoxicity. It is released in stress responses, overexpressed in immune-privileged sites, and is associated with recurrent miscarriage. Our evidence suggests a mechanism for inhibition of LAK cell cytotoxicity by MIF: through similarity between MIF and MHC class I. As such, MIF could act as a systemic (stress response) or tissue-wide (immune-privileged tissues) mechanism to inhibit NK cell function under the control of hormonal, psychological and inflammatory stimuli. Our observations about MIF add new information to the complexity of MHC class I proteins, their receptors, mimics and functions.

4.5. Figure Legends

Figure 4-1. MHC class I is purified by immunoaffinity chromatography with mAb OX18. (A) ELISA detects OX18-reactive material eluting from OX18 immunoaffinity column with 0.05 M diethylamine 0.5% triton X-100 650 mM NaCl pH 11.5. After extensive washing with column loading buffer, the column was sequentially eluted with four increasingly stringent buffers (15 volumes 10 mM Tris 0.5% triton X-100 pH 8.2 150 mM NaCl, 15 volumes 10 mM sodium phosphate 0.5% triton X-100 150 mM NaCl pH 10, 15 volumes 10 mM sodium phosphate 0.5% triton X-100 300 mM NaCl pH 10 and with 0.05 M diethylamine 0.5% triton X-100 650 mM NaCl pH 11.5. The majority of OX18-reactive material eluted with the final buffer. Fractions were neutralized with 1 M Tris-HCl pH 6.7, coated on ELISA plates and OX18 immunoreactive material detected with OX18 mAb, anti-mouse Ig-HRP and OPD (*o*-Phenylenediamine dihydrochloride) substrate. (B) Several proteins were purified by OX18 immunoaffinity. Fractions with the most OX18-reactive material (fraction 11-13) were combined and subjected to SDS-PAGE silver staining (left), western blot with OX18 anti-MHC class I mAb (center) and polyclonal anti-MHC class I Ab (right). Results are representative of four separate immunoaffinity purifications.

Figure 4-2. MHC class I and Macrophage Migration Inhibitory Factor are found in anti-MHC class I purified material. (A) Peptide sequences of MHC class I and MIF identified by MALDI-QTOF and Mascot search engine in fractions purified by immunoaffinity with OX18. (B) Identity of prominent bands in OX18 purified fractions. Results were obtained as in (A); results are representative of two experiments (A,B). (C) Both

MIF and β2m were detected by western blot in OX18 purified fractions; results are representative of three experiments.

Figure 4-3. MIF is bound by anti-MHC class I mAb and does not elute from isotype mAb immunoaffinity columns. (A) Recombinant MIF is bound by anti-MHC class I mAb OX18 but not other mAb that recognize conserved epitopes of MHC class I. Results are pooled from three independent experiments ($p > 0.05$, t-test, error bars represent standard error of the mean). (B) MIF does not non-specifically bind and elute from isotype mAb immunoaffinity columns. Isotype mAb column paired with anti-MHC class I immunoaffinity column (Figure 4-1) was loaded and eluted as in Figure 4-1. ELISA to detect MIF or MHC class I was performed as in Figure 4-1A. (C) In denaturing immunoblot, anti-MIF polyclonal antibody does not detect mouse MHC class I ($H2k^b$, right) and anti-MHC class I polyclonal antibody does not detect MIF (left). (D) OX18 and anti-MIF were confirmed to bind $H2k^b$ by ELISA (right). Background absorbance of wells without added primary antibody was subtracted from shown values. N=3, $p < 0.05$, error bars represent standard error the mean.

Figure 4-4. Amino acids forming an MHC class I epitope recognized by OX18 mAb and bound by NK cells are found in MIF. Key residues of an exposed loop of MHC class I α3 domain that contacts LILRB1 (bold black line) and OX18 mAb (bold red line) are found in MIF. LILRB1 contact residues in human MHC class I may be the same used by PIRB in mouse and represent a functionally accessible binding site on this region of MHC class I. (A) Amino acids implicated in OX18 binding of rat MHC class I are highly conserved. Amino acid substitutions are shown by inserted letter. RT1 proteins known to bind OX18 (top panel) are RT1.Ac (AAC52551), RT1.A2c (CAA62021), RT1.A2n (CAA62026), RT1.A1k

(CAB56231), RT1.A1n (CAA62025), RT1.E2 (CAD60945), RT1.U cc1 (CAA06295), and RT1.L2 (AAR96255). RT1.L1 mutations (R194D, G207D, and W243S) ablate binding of OX18. Five RT1 expression clones do not bind or weakly bind OX18 (RT1.U cc22 [CAA06297], RT1.U cc23 [CAA06298], RT1.Au [CAA57631], RT1 11/3R [CAA47838], RT1.L1 [AA085418]). These mutations suggest A244S may also ablate OX18 binding. Sequences were aligned with ClustalW[78] and edited with BioEdit. (B) The OX18 binding epitope of RT1.A is variably conserved by mouse MHC class I. Sequences were aligned using MultiAlin[79]. Neutral substitutions are shown in gray, non-neutral substitutions are shown in red. Substitutions cluster around LILRB1/OX18 binding sites. Sequences used for comparisons were H2D^{b} (1YN7A), H2D^{d} (NP_034510), H2K^{b} (NP_001001892), H2K^{d} (P01902), H2L^{d} (AAH92284), Qa-1 (AAD53968), Qa-2 (P14429), H2-T3 (P14432), and TL (CAA26586). (C) Cartoon representations of rat MHC class I RT1-Aa and human MHC class I HLA-A crystal structures (1ED3, 1I4F) showing amino acids important in binding LILRB1 and potentially OX18. The individual domains comprising MHC class I (α1, α2, α3 and β2m) are indicated. Amino acids implicated in forming the OX18 epitope on rat MHC class I are highlighted in red (193-198, G207, W243, A244). LILRB1 contact residues on HLA-A are highlighted in red (consensus[44] P193, I194, S195, D196, E198). LILRB1 and OX18 binding sites are located on similar loops and regions of MHC class I.

Figure 4-5. MIF-(9-18) forms part of a motif on MIF resembling MHC class I and bound by OX18. (A) Potential location of the OX18 binding site in MIF. The AB loop (193-198, PRPEG) associated with MHC class I binding of OX18 and LILRB1 (in human) is found in interrupted form in MIF (11-18, PRASVPEG). Sequences used were rat MIF (P30904), mouse MIF (NP_034928), RT1.Aa (P16391). Sequences were aligned with MultiAlin[79].

Sequences were truncated, and gap tolerance increased to demonstrate potential OX18 binding site. (B) Cartoon representation of the rat MIF crystal structure (1FIM) showing residues involved in binding OX18, and potentially receptors for MHC class I. The view is down the three-fold axis of the dimer. Amino acids involved in LILRB1 (in human) and OX18 binding that are also found in MIF are highlighted in red (P11, R12, P16, E17, G18). A loop in the linear structure PRASVPEG brings the OX18 and LILRB1 binding residues PRPEG into more continuous linear alignment. (C) OX18 binds MIF-(9-18). A peptide representing MIF-(9-18), NVPRASVPEG, and a scrambled and conservatively mutated version of this peptide SAGVDNAGKA were coated on 96 well plates for ELISA with OX18. Binding of isotype control mAb (IgG1) and OX18 mAb to MIF-(9-18) is expressed as a percentage of binding to scrambled peptide. $n=3$, $p<0.05$, error bars represent standard error of the mean.

Figure 4-6. MIF inhibits binding of H2D^{b} tetramers to LAK cells. Four day LAK cells were incubated 20 minutes with MIF or BSA (20 µg/mL) before addition of H2D^{b} tetramers and anti-CD3 mAb. (A) Representative flow cytometry analysis. Region R2 (center panel) represents gating on CD3$^{low/negative}$/ H2D^{b} tetramer^{high} cells, used below. Presence (+) or absence (-) of H2D^{b} tetramer is indicated above each scatter plot. (B) MIF does not inhibit binding of H2D^{b} tetramer or anti-CD3 mAb to CD3^{high} cells. (C) MIF inhibits binding of H2D^{b} tetramer to CD3$^{low/negative}$/H2D^{b} tetramer^{high} cells (region R2), but does not inhibit binding of anti-CD3 mAb to CD3^{high} cells, CD3$^{low/negative}$/H2D^{b} tetramer^{high} cells, or CD3^{high}/H2D^{b} tetramer^{high} cells. Error bars show standard error of the mean ($n=4$). * $p<0.05$.

4.6. Figures

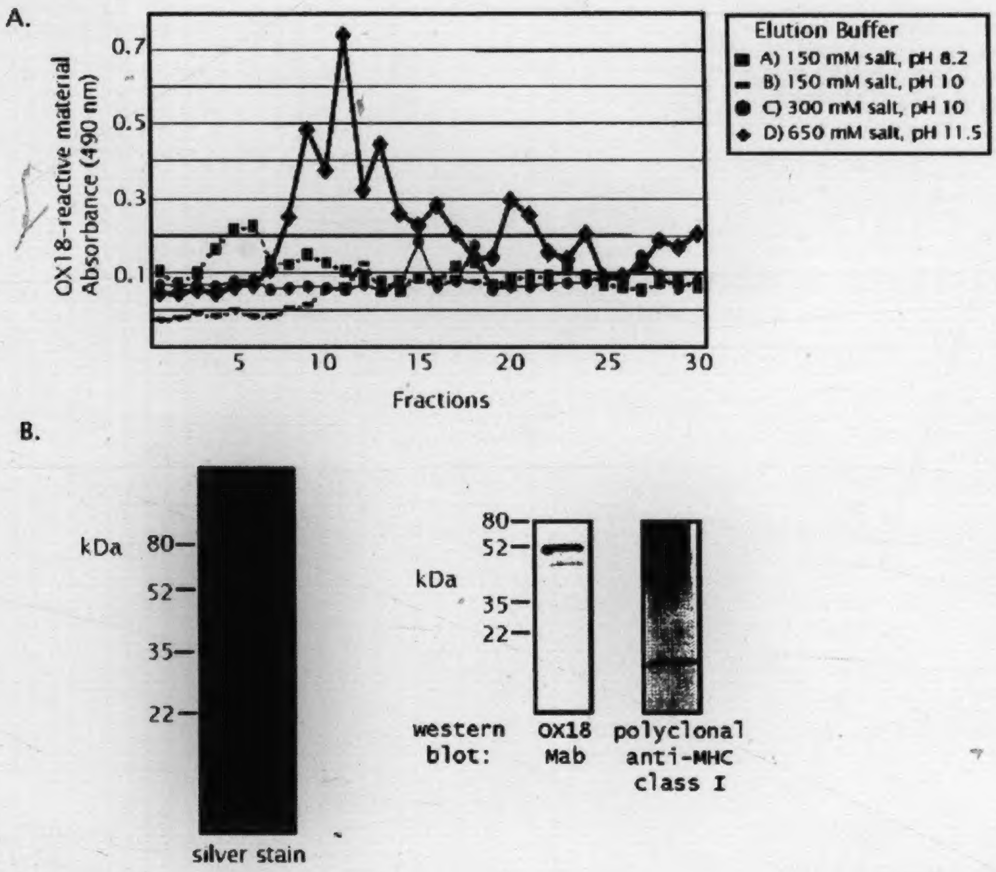


Figure 4-1. MHC class I is purified by immunoaffinity chromatography with mAb OX18.

A.

Identified Protein	Observed	Mr (expt)	Mr (calc)	Miss	Score	Rank	Peptide
MHC class I RT1.Au precursor (AAC32532)	361.76	721.50	721.40	0	33	1	YLELGK
	528.38	1054.74	1054.82	0	47	1	WASVVVPLGK
	533.31	1064.60	1064.46	0	46	1	EGPEYWER
	726.45	1450.89	1450.72	0	61	1	TWTAADFAAQITR
	816.01	1630.00	1629.80	0	27	1	FIAVGYVDDTEFVR
					Total Score	213 (scores >41 p<0.05)	
rat macrophage migration inhibitory factor (AAA62664)	419.31	836.60	836.45	0	28	1	LHISPDR
	523.87	1045.73	1045.56	0	47	1	LLCGLLSDR
	644.45	1286.89	1286.68	0	57	1	PMFIVNTNVPR
					Total Score	132 (scores >42, p<0.05)	

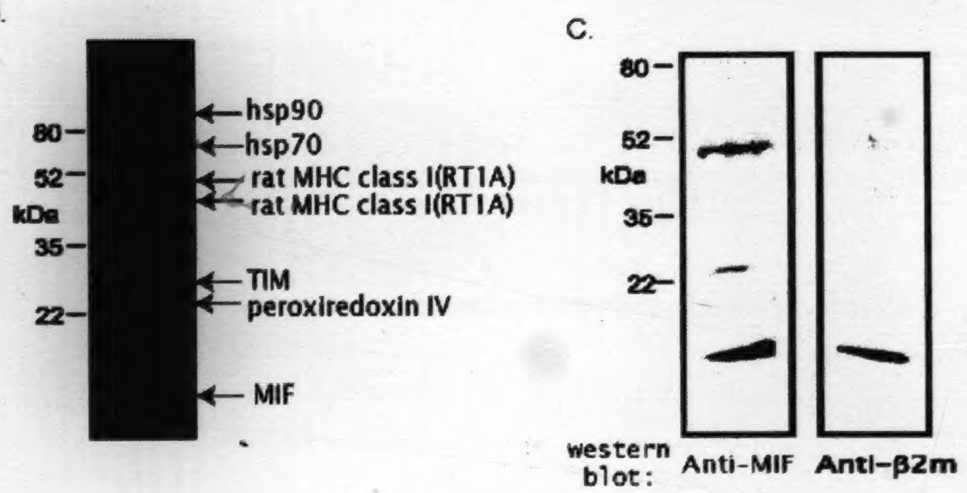

B.
80
52
kDa
35
22
hsp90
hsp70
rat MHC class I(RT1A)
rat MHC class I(RT1A)
TIM
peroxiredoxin IV
MIF
C.
80
52
kDa
35
22
western blot:
Anti-MIF
Anti-β2m

Figure 4-2. MHC class I and Macrophage Migration Inhibitory Factor are found in anti-MHC class I purified material.

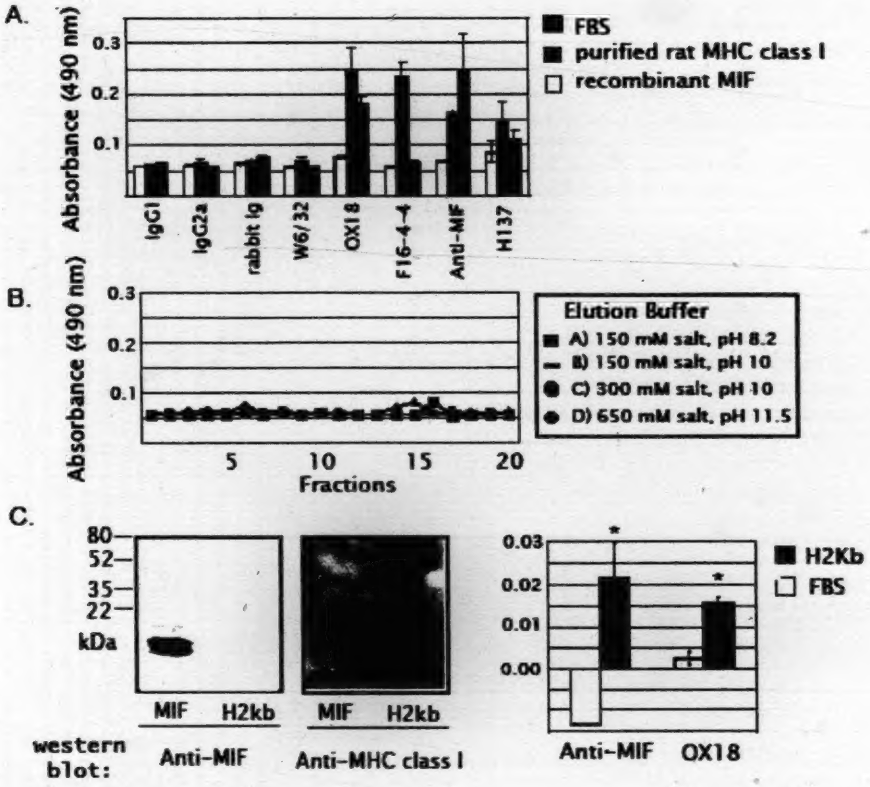
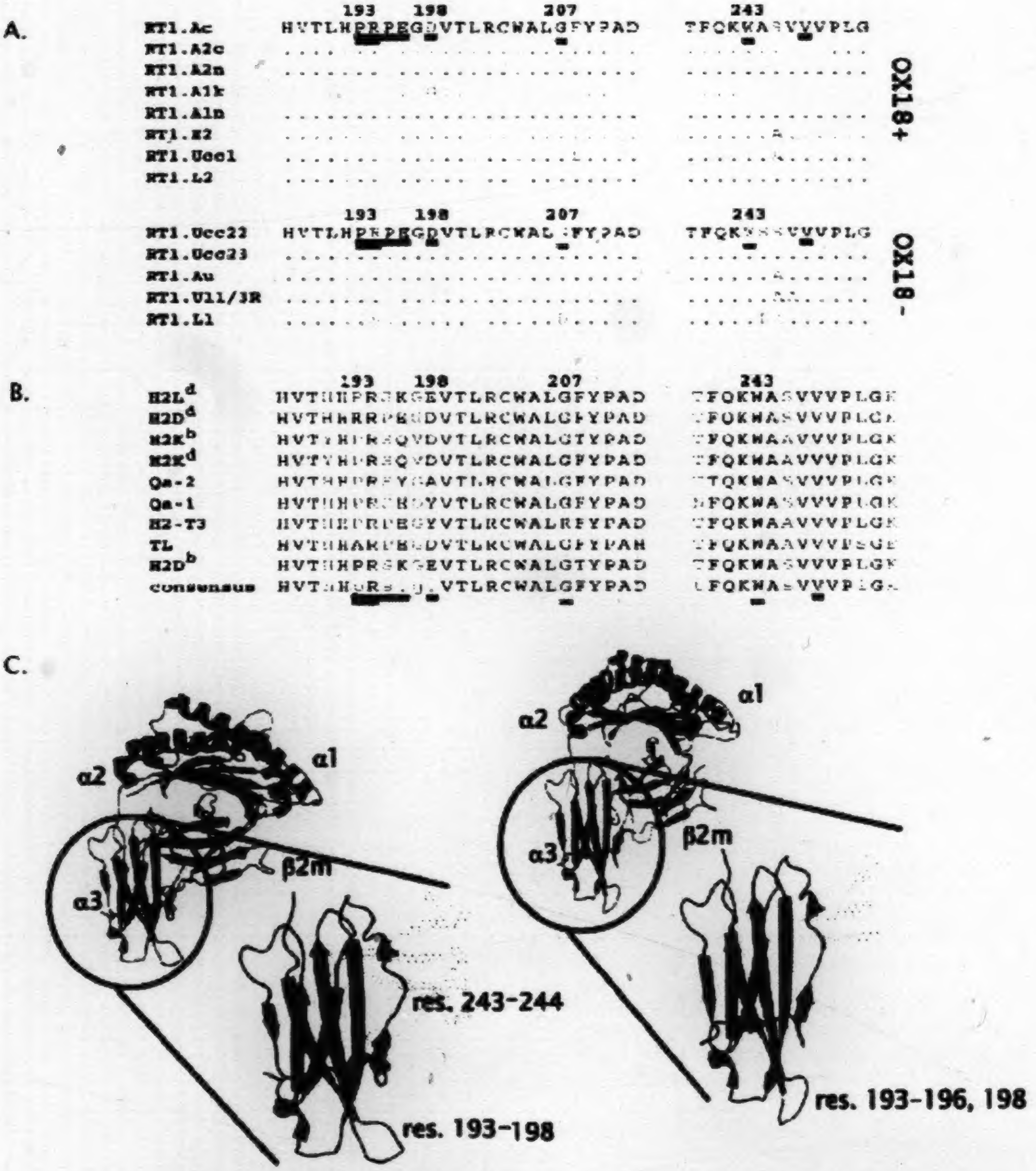


Figure 4-3. MIF is bound by anti-MHC class I mAb and does not elute from isotype mAb immunoaffinity columns.


A.
193
198
207
243
RT1.Ac
RT1.A2c
RT1.A2n
RT1.A1k
RT1.A1n
RT1.E2
RT1.Ucc1
RT1.L2
OX18+
RT1.Ucc22
RT1.Ucc23
RT1.Au
RT1.U11/3R
RT1.L1
OX18-
B.
H2L^d
H2D^d
H2K^b
H2K^d
Qa-2
Qa-1
H2-T3
TL
H2D^b
consensus
C.
α1
α2
α3
β2m
res. 243-244
res. 193-198
res. 193-196, 198

OX18 binding site
Rat MHC class I (RT1A.a)

LIILRB1 binding site
Human MHC class I (HLA-A2)

Figure 4-4. Amino acids forming an MHC class I epitope recognized by OX18 mAb and bound by NK cells are found in MIF.

A.

```
Mouse MIF    -VNT-NVPRASVPEGFLS
Rat MIF      -VNT-NVPRASVPEGFLS
RT1.Aa       HV-TLH-PR---PEG---
consensus    .VnT.nvPRasvPEGfls
```

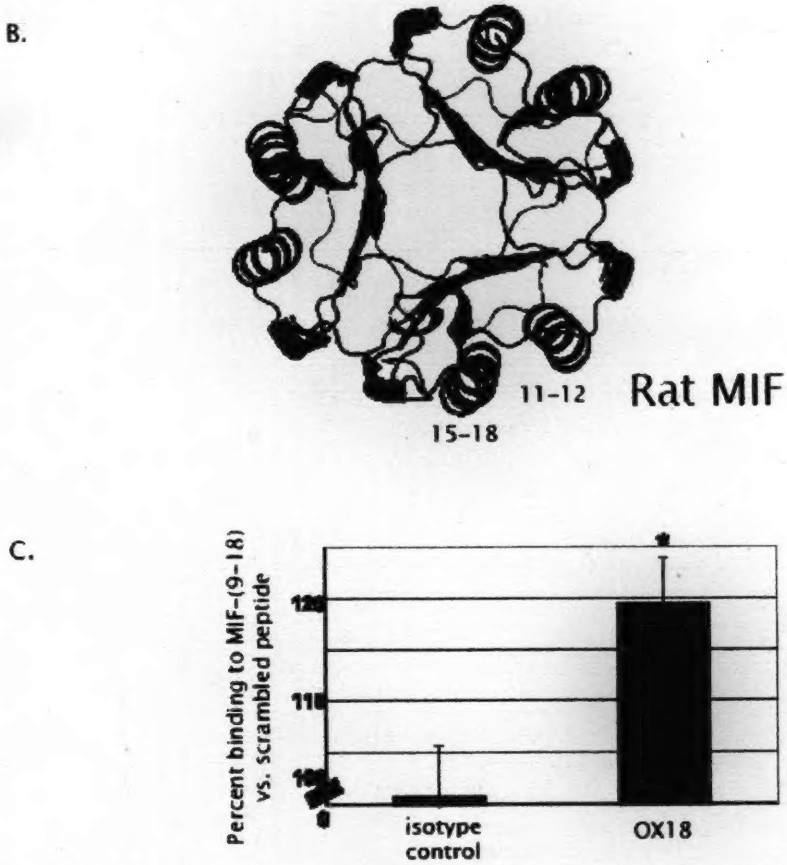


Figure 4-5. MIF-(9-18) forms part of a motif on MIF resembling MHC class I and bound by OX18.

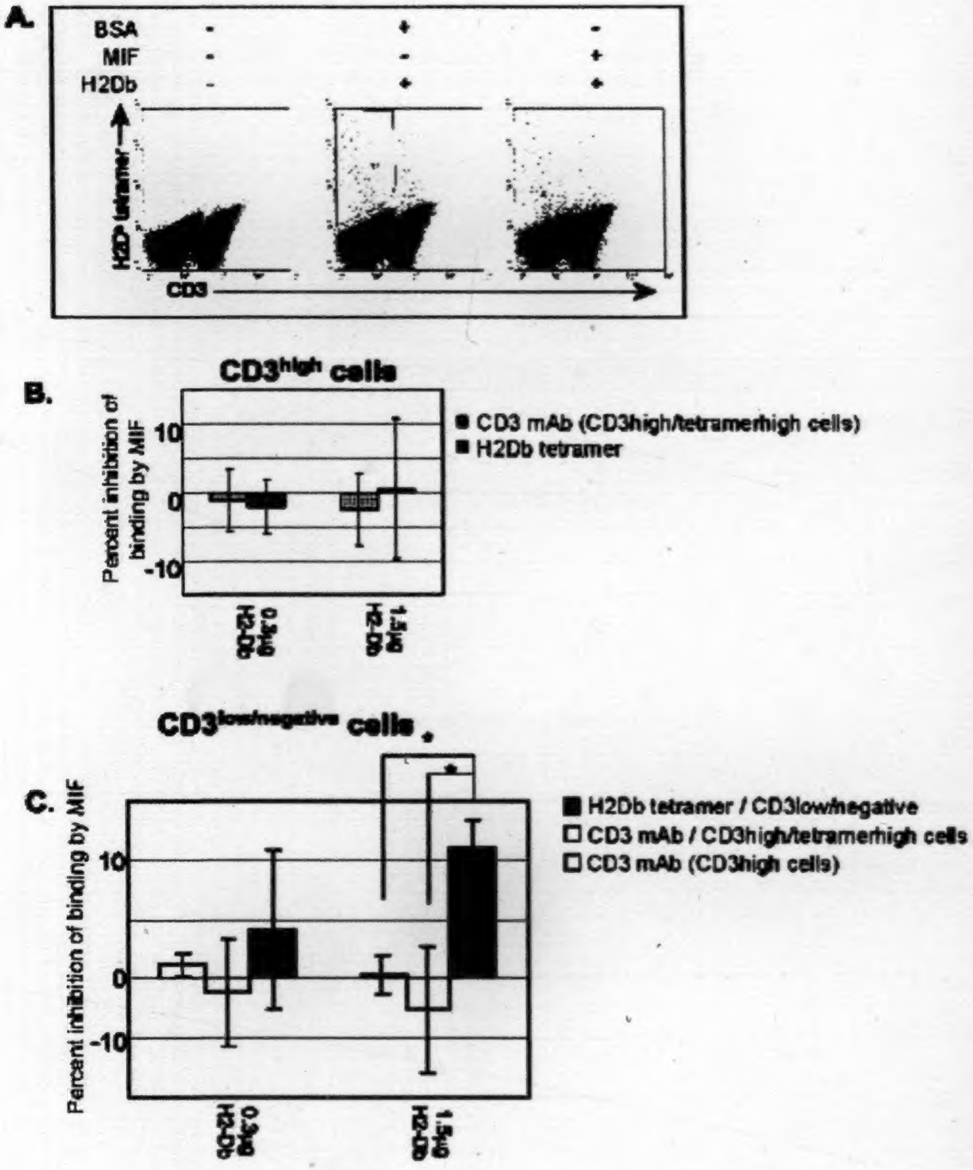


Figure 4-6. MIF inhibits binding of H2D^b tetramers to LAK cells.

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5. ANTI-RAT CD8α CLONE OX-8 BINDS UNIDENTIFIED PROTEINS AT 56-64 KDA, INCONSISTENT WITH CD8α

Clone OX-8 is a widely used mAb that binds rat CD8α [1]. OX-8 induces rat macrophages to produce TNF, IL-1β and nitric oxide in a Syk dependent manner [2;3]. Another anti-CD8α mAb (G28) used in published studies [2;3] did not bind CD8α on *ex vivo* rat macrophages. As such, the specificity of anti-CD8α OX-8 for CD8α was important to conclusions about CD8α on rat macrophages.

For the first time the anti-rat CD8α mAb OX-8 was used here in western blot for rat CD8α. While OX-8 identified a 37 kDa protein in splenic T cells by western blot, in agreement with immunoprecipitation data showing the Mr of rat CD8α [1], higher Mr bands (56-68 kDa) were also detected (Figure 5-1). Fifty-six to 68 kDa proteins bound by anti-rat CD8α mAb OX-8 were not mitochondrial, like the F1 ATP synthase detected by clone D9 (Figure 5-1). The higher Mr proteins detected by anti-CD8α OX-8 may be forms of CD8α that (re)aggregate due to characteristics not occurring in T cells, a novel form or modification of CD8α, or an unrelated protein. Surprisingly, OX-8 did not detect proteins within the predicted Mr of rat CD8α in rat alveolar macrophages, only high Mr proteins (56-68 kDa) were detected (Figure 5-1). This Mr difference may be attributable to N-linked glycosylation of CD8α (rat but not human CD8α is N-glycosylated), or OX-8 may cross-react with a protein that is not CD8α. Using the OX-8 mAb by immunohistochemistry others have observed cytoplasmic localisation of proteins recognized by OX-8 [4]. It is possible, even probable, that CD8 is present in endocytic or recycling vesicles as has been shown for lck, LAT and CD4 [5]. Given the uncertainty about the localisation and Mr of proteins detected by OX-8 mAb, particularly in macrophages, caution would be wise in interpreting experiments using this mAb.

Other data helps validate suggestions that CD8 protein is present and functional on rat macrophages. An anti-CD8β mAb elicited responses from rat macrophages similar to OX-8 suggesting that the release of TNF, IL-1β and nitric oxide observed with anti-CD8α mAb OX-8 does occur via CD8 [2;6;7]. Furthermore, a second anti-rat CD8α mAb (clone R1-10B5) bound rat macrophages (chapter 2) and inhibited binding of rat macrophages to MHC class I (Figure 2-6).

5.1. Figure Legend and Figure

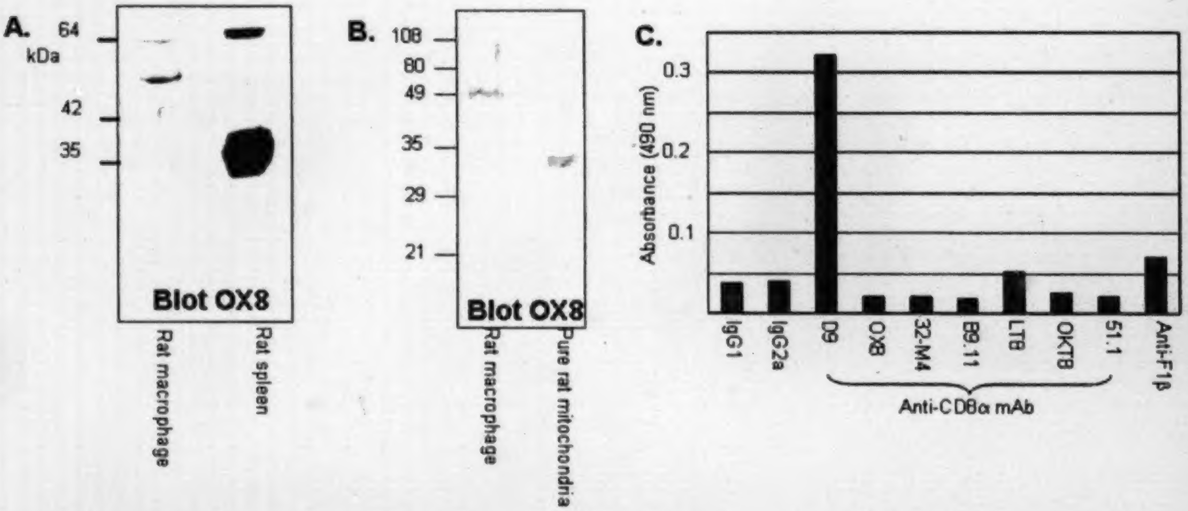

Figure 5-1. In macrophages anti-rat CD8α mAb OX-8 binds only proteins of significantly higher Mr than expected for CD8α. (A) While CD8α is described as a 34-37 kDa protein, anti-CD8α mAb OX-8 also binds 56-68 kDa proteins in rat macrophages and spleen cells (source of CD8α+ T cells). Only 56-68 kDa proteins are recognized in western blot with OX-8 in rat macrophages, suggesting that at least in denatured form OX-8 does not recognize CD8α in rat macrophages. (B) The 56-68 kDa proteins detected by OX-8 are not mitochondrial, or (C) associated with the F1 ATP synthase, like the 52 kDa protein detected by anti-human CD8α mAb D9. Purified rat mitochondria (B) from liver was a gift of Dr. Jean Vance (University of Alberta). In (C) crude mitochondrial preparations were immunoprecipitated with anti-F1 ATPase mAb beads according to supplier protocol (Mitosciences).

5.2. Literature Cited

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6. DISCUSSION

This thesis targeted gaps in our understanding of cell types that express CD8 and the functions of CD8 that might be independent of the TCR.

6.1. Species Differences in the Cell Types That Express CD8

The studies composing this thesis have contributed to understanding the expression of CD8 in two ways. For the first time human monocytes are demonstrated to express CD8α in a manner that renders it unlikely they acquire CD8 protein from other CD8+ cells by processes similar to "shaving" [1] or troglocytosis [2]. The latter was the case for reports of CD8β on mouse dendritic cells [3]. Together the presence of CD8α mRNA and CD8α protein in highly enriched (>99%) human monocytes and a human monocytic cell line (THP-1) demonstrates that human monocytes synthesize CD8α (Table 6-1). Levels of CD8α similar to that found on human monocytes are found on human macrophages, at least from the alveolar compartment. These cells may acquire CD8α from other CD8+ cells, synthesize their own CD8, or acquire soluble CD8α [4] from lung fluid.

6.2. Species Differences in CD8-Linked Signaling Proteins

Although CD8 is expressed on human monocytes and is relevant in human health, CD8 is absent on mouse monocytes [5]. Mouse and human species diverged about 65 million years ago and while mice and humans are genetically highly similar, pressures unique to their environmental niches have generated significant differences in their immune systems [6]. Table 1-2 summarizes several differences in CD8 between mice and human. CD8 is absent from mouse NK cells [7] but present on human [7-10] and rat NK cells [11;12]. Our evidence has solidified a pattern of species differences in the expression of CD8: NK cells and monocytes are CD8+ in human and rat, but CD8- in mice (Table 6-1). These observations suggest rat and human CD8 has functions distinct from mouse CD8 that enables its operation on monocytes and NK cells.

On the other hand, human and rat NK cells and monocytes may have unique receptors or signaling apparati that allow them to productively work with CD8. For example, Syk is expressed in some T cells (thymocytes and mature effector T cells) in humans but not in mice [6;13], and perhaps Syk in humans is capable of participating in signaling with TCR and CD8 in ways that mouse Syk cannot. If CD8 can co-activate Syk signaling with TCR in humans, it may be able to co-activate monocytes, macrophages and dendritic cells through some Syk-dependent receptors.

6.3. Species Differences in Genomic Regulation of CD8α Expression

There are differences between rat, mouse, and human in which cell types express CD8α and CD8β (summarized in Table 1-1, part I and II). Genomic elements controlling CD8α expression also exhibit species differences. *Alu* repeats are believed to be primate-specific, not being found in mice and rats, yet *Alu* repeats appear to be important for human CD8 expression [14] (Table 1-2). An inter-species study of CD8 expression on NK cells, monocytes and macrophages would help clarify the apparent species differences in cell types that express CD8.

CD8 expression appears to be largely controlled by regions distal to the CD8α and CD8β genes such as enhancers, matrix attachment sites, DNA methylation and chromatin accessibility (reviewed in [15]). These modifiers of larger DNA structures may allow co-expression of CD8 and functionally related genes. Four DNA hypersensitivity sites have been described around the human CD8α gene [14]. Two of these DNA hypersensitivity sites (HS1 between CD8α exon 4 and 5, and HS4 after the last exon of CD8α were not T cell specific, and linkage of the 14 kb region containing the four DNA hypersensitivity sites to the CD8α gene is sufficient to allow CD8α expression in NK cells [7]. Thus sites that may control CD8α expression in cells other than T cells have been identified. A different number of DNA hypersensitivity sites were noted for CD8α in mice and human studies [15], suggesting further species differences in expression of CD8 (Table 1-2). Interestingly, Ikaros can activate transcription of CD8α and may initiate many epigenetic modifications of the type involved in CD8α transcription [16]. Ikaros also appears to be present in monocytes, and can be upregulated in dendritic cells [17]. Thus, Ikaros may be one factor that regulates CD8α expression in monocytes and dendritic cells. Differential expression of factors like Ikaros among cell types may lead to differential expression of a subset of genes, including CD8 on monocytes in some species.

6.4. CD8α on Monocytes and Macrophages Binds MHC Class I

As previously discussed, CD8α on rat macrophages was not bound by an anti-CD8α mAb G28 that binds the Ig-V-like MHC class I binding region of CD8α [12;18]. This suggested the possibility that CD8α on rat macrophages may bind MHC class I with a different affinity or avidity, or potentially not at all as compared to CD8α on T cells. Given the differences in CD8α on human monocytes and T cells as seen by 2-D electrophoresis, and their differences in binding anti-CD8α mAb clone D9, it was possible that CD8α on human monocytes also varied in a way that could affect its binding to MHC class I. Thus, I tested whether CD8α on human monocytes and rat macrophages binds MHC class I.

A potential complicating factor in the measurement of CD8-dependent adhesion of rat macrophages to MHC class I was the presence of MIF in enriched rat MHC class I preparations. As demonstrated in Chapter 4, the mAb used to purify rat MHC class I cross-reactively bound MIF, and co-purified MIF with MHC class I. The adhesion of a rat macrophage cell line (NR8383) to the preparations containing MIF and MHC class I was inhibited by anti-CD8α mAb after 1 hour at 37°C. Hypothetically, binding of rat macrophages to MIF, not MHC class I, could be inhibited by anti-CD8α mAb in these experiments. To test this possibility we performed the same adhesion assay with amounts of recombinant mouse MIF (>99% conserved with rat MIF) equivalent to the amount in enriched MHC class I preparations used. Despite our anticipation that rat macrophages might bind recombinant MIF, we did not detect adhesion of rat macrophages to MIF above background adhesion or an effect of anti-CD8α mAb (not shown). Several explanations for the inability to detect rat macrophage adhesion to MIF are possible. The rat macrophage cell line used may not have receptors or other factors that render them susceptible to MIF, or adhesion of rat macrophages to MIF may be masked by background macrophage adherence through other mechanisms. Whatever the reason that rat macrophage adhesion to MIF was not detected, these experiments suggest macrophages bound in a CD8α-dependent manner to MHC class I, not MIF.

Binding of HLA-A2 tetramers to human monocytes was partially inhibited by anti-CD8α mAb. Rat macrophages bound rat MHC class I coated on microwells, and this binding could be inhibited by anti-CD8α mAb. However, it remains possible that treatment of rat macrophages with anti-CD8α mAb at 37 C for 1 h induces a change in these cells that alters their ability to bind MHC class I, in a way unrelated to CD8 [19]. This is less likely in experiments with human monocytes using anti-CD8α mAb to inhibit binding of soluble MHC class I tetramers at 4 C in the presence of sodium azide, as most cellular changes are significantly reduced in these conditions.

On T cells, anti-CD8α mAb only inhibits binding of MHC class I tetramers if the MHC class I is complexed with a peptide recognized in an antigen-specific manner by the TCR [20;21]. While more elaborate explanations are imaginable, the ability of anti-CD8α mAb to inhibit binding of MHC class I tetramers may be most simply explained as either the ability of CD8α to promote binding of TCR to MHC class I, or to stabilize binding of tetramers where TCR and CD8 bind independent MHC class I molecules in the tetramer. A similar situation is possible on monocytes. CD8α may promote binding of MHC class I tetramers to another receptor, such as ILT family receptors, via mechanisms suggested in the section below "CD8: Re-examining the Co-receptor Concept". Alternatively, CD8α and other MHC class I receptors, like ILT family receptors may bind to separate MHC class I molecules in multi-valent tetramers. Either case may be observed as inhibition of binding of MHC class I tetramers to monocytes with anti-CD8α mAb.

CD8αα binds MHC class I (e.g. [22]), and CD8αα enhances binding of MHC class I tetramers to TCR [23]. At present, data suggests CD8β is required for CD8 to bind MHC class I tetramers in the absence of the TCR, from one example with mouse immature thymocytes [24]. Human monocytes express only CD8α, and thus presumably CD8αα homodimers, suggesting from current knowledge [20;21;23;24], that CD8 on monocytes should not bind MHC class I tetramers alone. If anti-CD8α mAb inhibits binding of MHC class I tetramers to CD8α alone on monocytes, and does not, as suggested above, prevent

CD8α from promoting or stabilizing binding of MHC class I tetramers in conjunction with a second receptor, CD8αα on monocytes may have a higher affinity or avidity for MHC class I than CD8 on mature T cells. I have discussed how CD8 binding to MHC class I varies between immature and mature thymocytes, before and after cell activation of CTL clones or blood T cells. Current data suggests CD8β is necessary for CD8 to bind MHC class I tetramers without a second MHC class I binding receptor [24]. Given the numerous cases in which CD8 binding to MHC class I is altered, it is possible a novel change in CD8αα on monocytes may allow CD8 to bind MHC class I tetramers without expression of CD8β.

Studies in this thesis strongly suggest CD8αα on human monocytes binds MHC class I, and suggest that CD8α on rat macrophages can also bind MHC class I. However other questions remain to be resolved. NK cells and potentially monocytes have several receptors with binding sites on MHC class I that do or do not overlap with CD8 [25]. Does low affinity CD8 binding to MHC class I promote binding of other receptors on monocytes to MHC class I? Does the glycosylation of CD8α on monocytes, and potentially each CD8+ cell type (NK cells, dendritic cells) vary in a way that affects its affinity or avidity for MHC class I?

6.4.1. Some Anti-CD8α mAb Clones Do Not Bind CD8 in Certain Circumstances: Relation to MHC class I Binding

Several studies have demonstrated that binding of certain anti-CD8α mAb differs between types of CD8+ cells but no explanation for this has been published [26-29]. Our laboratory previously described that one of two anti-CD8α mAb (G28), binds rat T cells but not rat macrophages [18], an observation since confirmed by others [30]. In contrast the same anti-CD8α mAb (G28) binds to rat monocytes [31;32]. Here, I have found that an anti-CD8α mAb (clone D9) binds human monocytes and CD8+ T cells retrieved from pathophysiological lung of some patients, but not peripheral blood T cells. Interestingly, clone D9 inhibits binding of MHC class I to monocytes, and clone G28 binds the Ig-like region of CD8α involved in binding MHC class I [12]. The differences that control mAb binding to CD8 Ig domain on monocytes and macrophages may influence CD8 binding to MHC class I.

6.4.2. Glycosylation and Inter-Molecular Interactions of CD8

An association of CD8 with another molecule may mask the epitope that D9 binds on CD8α on peripheral blood T cells. CD8α can associate on the same cell with MHC class I [33-35]. This may mask the MHC class I binding site on CD8α and prevent CD8α binding to MHC class I on an opposing cell, thereby blocking CD8-mediated cell activation. Activation of the NK cell receptor Ly49A is regulated in this way [36]. *In cis* interaction of Ly49A with MHC class I, impedes Ly49A binding to MHC class I *in trans*, on a target cell, thereby preventing NK cell activation. Regulated binding of CD8α to MHC class I in the plasma membrane of the same cell may prohibit binding of anti-CD8α mAb clone G28 to rat macrophages and allow binding of anti-CD8α mAb clone D9 to human monocytes.

Another intriguing case where changes in binding of anti-CD8α mAb may be related to changes in the ability of CD8 to bind MHC class I was recently described. Calcium or its sequestration dramatically altered the binding of some anti-CD8α mAb to T cells, without CD8 internalization [37]. Calcium is known to bind sialic acid [38;39], likely due to their opposing charges. Sialic acid on the stalk region of CD8β affects its ability to bind MHC class I tetramers [24]. Therefore, calcium may bind sialic acid on the CD8α [40] or CD8β stalk region and alter the ability of anti-CD8α mAb to bind. If the negative charge on sialic acid contributes to its inhibitory effect on CD8 avidity for MHC class I tetramers, the addition of calcium or calcium influxes during T cell activation may neutralize or potentiate this charge, and enhance or reduce CD8 binding to MHC class I at the precise times and locales of TCR activation. Calcium (1 mM $CaCl_2$) or its sequestration with EDTA had no effect on binding of anti-CD8α mAb D9 to lymphocytes (data not shown).

It is intriguing that many of these differences among cell types or environments in binding of anti-CD8α mAb suggest that there are changes in the ability of CD8 to bind MHC class I. However, evidence of this is lacking at present.

6.4.3. The Possibility of Structural Alternations in CD8: Disulphide Bond Switches

A second possibility that could explain differential binding of anti-CD8α mAb to CD8 on different cell types is a change in CD8 structure or orientation. Subtle changes in conformation of MHC class I or CD8 affect CD8 binding to MHC class I [22;41]. A select few anti-CD8α mAb enhance binding of MHC class I tetramers, perhaps by inducing a conformational change in CD8 that enhances CD8 binding to MHC class I [21;42]. No structural differences in CD8 by itself or bound to MHC class I are observed in crystal structures, with one exception (discussed in [43]). This suggests, as one interpretation, that different conformations of CD8 can be induced, perhaps physiologically, that change CD8 affinity or avidity for MHC class I.

Adhesion of CD8 before and after TCR activation does not rely on all the same residues in MHC class I [44;45]. While CD8 binding to MHC class I depends on charged amino acids in the entire length of a loop of the MHC class I α3 domain, only one residue in this loop was required for CD8 binding to MHC class I after TCR stimulation [44]. Significant avidity-enhancement of CD8 may minimize the need for multiple contacts with MHC class I [44]. Another possibility is a structural change in CD8 that changes the residues CD8 contacts on MHC class I and thereby CD8 binding affinity [44].

Changes in disulphide bonds may be incipient actors in alternate conformations of CD8. Most Ig superfamily members contain two cysteines in their Ig domains, one in each of the B and F strands that form a disulfide bond with a conserved geometry [46]. In contrast the CD8α Ig domain contains three cysteines on B, F and C strands (Figure 6-1A) in mouse, rat and human.

What may be the role of this extra cysteine residue, that is not found in canonical Ig superfamily members, yet is conserved in rat, mouse and human CD8α? Intriguingly, an analogous free cysteine residue is found in proximity to the disulfide bond in domain 2 of CD4. In domain 2 of CD4, switching of a B-C, non-canonical disulphide bond (steady state) to a B-F disulfide is catalyzed by thioredoxin, but not other thiol redox enzymes [47]. This disulfide bond switch in CD4 changes its affinity for gp120 of HIV-1 and promotes HIV-1 infection of T cells [47].

Contradictory results concerning which cysteines (B-C or B-F) form disulphide bonds in the Ig domain of CD8 suggest that a disulphide switch mechanism exists in CD8. Two laboratories found that the intrachain disulfide bond in the CD8 Ig domain is formed non-canonically between B and C strands, like CD4 [47], rather than B and F strands [48;49]. In contrast crystal structures of mouse and human CD8α have visualized the canonical Ig domain B-F disulfide bond (Figure 6-1) [50-52]. It is possible, despite the use of iodacetamide in cell lysis buffers to block free cysteines, that an aberrant disulfide bond between B and C strands was induced during CD8 purification by both groups that found the non-canonical disulphide bond [48;49]. The distance between B and C cysteines in crystal structures of CD8α is slightly longer (8.2-8.4 angstroms) than disulphide bonds in most Ig domains (6.6-7.4 angstroms), suggesting that formation of this bond would imply a significant adjustment in CD8 structure (Figure 6-1) [52].

Mutation of B or F cysteines minimizes expression of CD8, suggesting a canonical B-F disulfide bond is the major disulphide bond in the CD8 Ig domain [43]. Mutation of the C strand cysteine appeared to increase the proportion of CD8αβ heterodimers [43] suggesting it is usually less important for CD8 structure but may influence CD8 heterodimer stability. Unfortunately, nothing is known of the influence of B, F, or C strand cysteines on CD8 binding to MHC class I.

Switching between B-C and B-F disulphide bonds in CD8α may alter CD8 affinity (like CD4 or integrin affinity activation), function, or binding to anti-CD8α mAb [48-52]. Given that different cell types probably express or localize to environments with different repertoires of thiol redox enzymes one might expect some variation in which disulphides are formed in sensitive proteins, like CD4 or CD8 by different cell types. In fact, novel disulphide bonds were recently reported in CD4 on monocytes compared to lymphocytes [53]. Thiol redox control of CD8 function would allow its adaptation to the intensity of an ongoing immune response by reflecting the inflammatory-redox setting of the local environment.

6.5. Post-Translational Modification of CD8α on Human Monocytes and T Cells

Sialylation regulates CD8 binding to MHC class I tetramers [24], and palmitoylation regulates the ability of CD8 to co-activate TCR responses [54]. Both CD8α [40;55] and CD8β [54;56] are sialylated and palmitoylated. The expression of enzymes involved in glycosylation or palmitoylation change depending on cell type and state of activation. Thus, monocytes and T cells may glycosylate or palmitoylate CD8α differently.

We have begun to characterize molecular differences between CD8α on human monocytes and T cells. CD8α was detected at 32 and 34 kDa with spots at several pI (range 6-9) at each molecular mass. Monocytes selectively lacked sialylated 34 kDa forms of CD8α visualized in T cells. The heterogeneity described by 2-D electrophoresis of CD8α here, and by others [57;58] or by mass spectrometry and chromatography [55;59] is much more complex than an on/off switch of sialylation. These changes in human CD8α have not been correlated to the expression of particular glycosylation enzymes to the same extent as in mouse [24;56]. Further study with more deglycosylating enzymes, followed by analysis of the glycosylation enzymes expressed in monocytes and T cells may elucidate the molecular differences noted between monocyte and T cell CD8α. While the ability of anti-CD8α mAb D9 to bind monocytes but not T cells from human blood was not explained by sialylation, it may be due to other O-glycosylation of CD8α or differences in which of the 10 potential sites of O-glycosylation in CD8α are used.

6.5.1. Differences in Glycosylation or Palmitoylation May Account for 32 and 34 kDa Mr of CD8α

The difference between 32 and 34 kDa forms of CD8α may be due to glycosylation, as the difference was eliminated with trifluoromethanesulfonic acid by others [60]. However, trifluoromethanesulfonic acid is supposed to remove all glycosylation [61], and thus should leave a form of human CD8α of 26-27 kDa, not 32 kDa [55]. Palmitoylation can cause small molecular mass shifts on SDS-PAGE gels, and CD8α is palmitoylated [8;62]. It is possible the acyl linkage of palmitoylation may be broken by trifluoromethanesulfonic acid, and the difference between 32 and 34 kDa forms of CD8α may be palmitoylation.

If we accept, despite evidence and opinion to the contrary (reviewed in [63])[64], that lipid rafts exist and are important for TCR activation, the ability of CD8αα on monocytes to be palmitoylated and associate with lipid rafts may be important for the suggested participation of CD8 in FcR signaling. However current literature suggests only CD8αβ associates with lipid rafts [65], and not CD8αα, as we propose is found on monocytes. For example, palmitoylation of mouse CD8β may explain CD8αβ's heightened ability to associate with lipid rafts, lck [23] and LAT [66], and elicit T cell activation compared to CD8αα homodimers [54;65]. However, similar experiments suggest both CD8α [55;67] and CD8β [54;65] can be palmitoylated, and both CD8αα and CD8αβ associate with lipid rafts [54;62]. [54;68;69] Whatsmore, while CD8β palmitoylation on one cysteine is proposed to drive CD8 association with lipid rafts [54;65], palmitoylation of at least three, and as many as seven cysteines may contribute to CD8 association with lipid rafts (Figure 6-2, two each in lck and LAT [70;71], three in CD8α, and only one to two in CD8β). Furthermore, CD8β (RRRAR) and CD8α (RNRRR) have motifs, resembling a sequence in CD4 (RHRRR) that was more important for CD4 association with lipid rafts than CD4's palmitoylation at a CD8β-like CVRC motif [72] (Figure 6-2). These charged sequences in CD8α and CD8β are highly conserved across species (Figure 6-2) and the association of CD8 with lipid rafts is likely much more complex than a single palmitoylation site in CD8β. While it has not been examined in this thesis, CD8αα on human monocytes and other cells may associate with lipid rafts.

In summary, CD8α may vary in glycosylation and palmitoylation among monocytes, T cells and other CD8+ cell types. This may modulate the ability of CD8 to induce intracellular signaling and activation, by changing its binding to MHC class I and strength of association with lipid rafts.

6.5.2. Dynamic Palmitoylation of CD8α May Prevent Binding of Lck and LAT

Palmitoylation, unlike myristolation and other lipid modification of proteins is not a static phenomenon. After ligation of several receptors palmitoylation and depalmitoylation of proteins may rapidly occur via acylthioesterases and acylthiotransferases [73]. For example, activation of BCR induces palmitoylation events promoting its association with lipid rafts [74], and this activated palmitoylation can be inhibited by oxidative stress [75]. Changes in palmitoylation and lipid raft association of CD8α [55;62] or CD8β [54] could rapidly change via acylthioesterases in the plasma membrane [73], or synthesis of differently palmitoylated CD8 because of the relatively short half-life of CD8α [55]. This could control partitioning of CD8 into lipid rafts, and regulate CD8 avidity [45;76;77] (as occurs with integrins [78]), and CD8 recruitment to FcR or TCR signaling complexes.

There is no consensus sequence for palmitoylation of transmembrane proteins [79] and transmembrane proteins can be palmitoylated on available cysteine residues just inside or within 12 amino acids of the membrane [79]. Two of the three cysteines available for palmitoylation of human CD8α [55;67] form the CxC motif necessary for interaction with lck/LAT (Figure 6-2)[66;80]. Thus, regulated palmitoylation of CD8α may exclude CD8 binding to lck or LAT. This could allow functions of CD8 to be separately engaged (Figure 6-3). CD8, palmitoylated and not bound to lck or LAT may associate with lipid rafts, and bind MHC class I but be unable to signal. In an unpalmitoylated form, CD8 may signal through lck or LAT, but associate with lipid rafts to a lesser degree (Figure 6-3). A few of the hypothetical possibilities for CD8 palmitoylation, association with lck/LAT and association with lipid rafts are presented in Figure 6-3. As LAT, lck, CD8α and CD8β are all palmitoylated, acylthiotransferases and acylthioesterases may control their lipid raft targeting, and signaling functions differently in each cell type, or in certain states such as T cell anergy.

6.6. The Problems and Benefits of Monoclonal Antibody Cross-Reactivity

Because the binding sites of TCR and Ab substantially adapt to the shape of a provided MHC class I-peptide complex or antigenic molecule [81-83], with what is called induced-fit binding, each TCR or Ab can bind multiple MHC class I-peptide complexes or pathogenic epitopes [84]. It is hypothesized that each TCR or Ab is capable of binding multiple ligands to help them keep pace with rapidly evolving pathogens [84]. Unfortunately this means that when mAb are used as experimental tools to draw conclusions about a single protein they will be prone to error. One basic way to guard against this source of error is to use more than one mAb that recognizes a target protein.

Ab usually establish contacts with 9-15 amino acids in a target protein, but the contact surface area is relatively small (~750 A°) compared to receptor interactions like CD8 or Ly49 with MHC class I (~2000 A°) [81]. Because of this, as a matter of statistical probability mAb may recognize "cross-reactive" proteins that are biologically and functionally unrelated. However, mAb could also be considered as an innovative tool for cell (surface)-wide searches for the identification of small functionally important regions of similarity not identified by sequence analysis. Potential examples of both of these possibilities were found in this thesis.

6.6.1. Some Anti-CD8α mAb Bind Proteins That are Not CD8α

6.6.1.1. Anti-CD8α Clone D9 Binds F1 ATP Synthase β Subunit

Anti-CD8α mAb D9 detected a 52 kDa protein by western blot; CD8α is a 32-34 kDa protein in humans. We considered the possibility that a novel form of CD8α may be detected by anti-CD8α mAb D9 at 52 kDa. Analysis of published northern blot data [4;85] and the genomic region surrounding CD8α had suggested the existence of upstream start codons leading into in-frame open reading frames contiguous with CD8α. However, these were of insufficient length to account for a Mr shift from 32 to 52 kDa. Likewise, human CD8α lacks a site for N-glycosylation, and is not N-glycosylated [55] suggesting N-glycosylation could not account for a Mr shift of 32 to 52 kDa. Careful analysis demonstrated that anti-CD8α clone D9 bound CD8α at 32 kDa and a protein at 52 kDa, the F1 ATP synthase β subunit, with no evident sequence (analysis with ClustalW) or structural homology (search for structural neighbors with VAST [Vector Alignment Search Tool]). No suggestion that F1 ATP synthase and CD8 were physically associated or functionally interrelated was found in the literature. Thus induced fit binding of clone D9, typical of many antibodies [83], may allow it to bind an unknown region of F1 ATP synthase with similarity to CD8α.

Taken together, the effects of anti-CD8α clone D9 on monocyte activation and MHC class I binding are likely to be mediated by CD8α and not the F1 ATP synthase β subunit. First, the F1 ATP synthase β subunit could not be detected on the monocyte surface, and therefore would be unlikely to influence monocyte function in the performed experiments. Second, other anti-CD8α bound monocytes at similar levels, and had similar effects on monocyte MHC class I binding (clone B9.11) and activation (clone 32-M4). Thus, despite the ability of anti-CD8α mAb D9 to bind the F1 ATP synthase β subunit, D9 also binds CD8α, and it is this ability to bind CD8α that likely enables the binding of clone D9 to monocytes and its effects on monocyte function.

6.6.1.2. Anti-Rat CD8α Clone OX-8 Binds CD8α and Unidentified Protein(s) of 56-64 kDa

Clone OX-8 is widely used to detect rat CD8α [86]. Many studies that have described CD8α+ rat macrophages have relied on clone OX-8 to detect CD8α, in part because another commonly used anti-CD8α mAb (G28) does not bind rat macrophages [87]. Moreover, many of the functions of CD8α on rat macrophages have been described using clone OX-8 [18;87]. As such, the specificity of anti-CD8α clone OX-8 for CD8α was important to conclusions about CD8α on rat macrophages.

While clone OX-8 has been demonstrated to bind CD8α by immunoaffinity purification and amino acid sequencing of CD8α [86], and by classical transfection studies with CD8α cDNA [49], use of OX-8 in western blot to detect rat CD8α had not been published prior to this thesis. OX-8 detected only proteins of higher Mr than CD8α (56-64 kDa versus 37 kDa for CD8α), in rat alveolar macrophages suggest OX-8 may recognize a protein other than CD8α on rat macrophages.

Other data helps validate suggestions that CD8 protein is present and functional on rat macrophages. An anti-CD8β mAb elicited responses from rat macrophages similar to OX-8 suggesting that the release of TNF, IL-1β and nitric oxide observed with anti-CD8α mAb OX-8 does occur via CD8 [18;26;88]. Furthermore, a second anti-rat CD8α mAb (clone R1-10B5) bound rat macrophages and inhibited binding of rat macrophages to MHC class I. Due to the consistency of effects on macrophage functions among anti-CD8 mAb, it is likely that anti-CD8α clone OX-8 binds native CD8α on rat macrophages. However, in some circumstances, such as in the denaturing conditions of western blot, OX-8 may bind proteins other than CD8α.

The flexibility of induced-fit binding exhibited by antibodies [83] may allow a mechanism to deal with pathogen mutation, but at the same time it may problematize the use of mAb in research to identify and study proteins. Studies using transfected cells to study CD8α function, for example, in cells where FcR signaling can be re-constituted by transfection [89], offer a complementary tool to identify CD8α-specific functions.

6.6.2. Consequences of a Small MHC Class I-Like Motif In MIF

MIF bound a monoclonal antibody (OX18) that recognizes a conserved epitope of all rat polymorphic MHC class I. Through sequence analysis a 10 amino acid sequence was identified that resembles MHC class I, and weakly bound OX18. A small MHC class I-like motif in MIF may be an accident of evolution with no functional consequences. However, both the OX18 epitope of MHC class I and MIF are implicated in the inhibition of NK cell cytotoxicity, suggesting the similarity between MIF and MHC class I may be physiologically relevant. Studies to demonstrate the physiological relevance of the MHC class I-like motif in

MIF will require a better definition of the region of similarity and the receptor that may interact with MHC class I and MIF.

6.6.2.1. Reducing MHC Class I to Motifs: Polymorphisms and Appropriations

Rather than each protein performing one function as once hypothesized, many proteins have multiple interactions and often multiple functions, associated with separate regions of their structure. MHC class I may be a prime example of one protein with many interactions. Many distinct surfaces of MHC class I are contacted by different receptors to generate or inhibit immune responses [90].

Several examples of viral and host homologues of MHC class I lack domains of classical MHC class I yet still control immune responses through receptors on NK cells or other cells (Figure 6-4). For example MIC-A and MIC-B are human MHC class I homologs expressed in a restricted manner, that do not associate with β2m [91;92] but control NK cell cytotoxicity [93]. Mouse MHC class I-like proteins Rae1-β and H60 contain only α1 and α2 regions of MHC class I [94] and are ligands for NKG2D (Figure 6-4). Similarly the non-classical MHC class I molecule T22 lacks a significant part of the α2 domain yet binds γδ TCR [95]. While some viral homologs of MHC class I in human, murine and rat cytomegalovirus and molluscum contagiosum virus retain the MHC class I α1,2 and 3 domains, the murine cytomegalovirus MHC class I homolog Unique Long Region Protein (UL)18 is missing a substantial part of the α2 domain (Figure 6-4) [96-98] [99]. With a high degree of selective pressure on MHC class I and MHC class I-like genes exhibited by their rates of gene duplication, polymorphism, appropriation and conservation, more homologues are bound to be identified. As small fragments of some proteins, like MHC class I, are by themselves functional domains, these fragments of proteins transposed into a second protein, as hypothesized for the MHC class I-like motif in MIF, may retain their function.

6.6.2.2. MIF Has Several Functional Domains, One of Which May Have Similarity to MHC Class I and Bind NK Cell Receptors

Data elucidating the importance of MIF in macrophage function, innate immunity, cancer, asthma, and angiogenesis is rapidly accumulating (reviewed in [100]). However, at least three distinct regions of MIF may mediate distinct biologic functions (Figure 6-5). In most cases it is not clear which region/molecular mechanism of MIF is responsible for the many signaling, cellular and organism-level effects of MIF.

The three functional regions of MIF are as follows. First, a receptor, CD74, has been demonstrated for MIF [101] and loops on the surface of MIF may mediate binding to CD74 (Figure 6-5)[101]. However, MIF also appears to be endocytosed independently of a receptor and interacts with several proteins including a member of the proteasome Jab1 [102], and MLCK [103]. These functions of internalized MIF are due to a second functional region of MIF, a thioredoxin activity associated with a CXXC motif. The thioredoxin motif of MIF is located in a peptide on the side of the MIF barrel trimer structure, distant from the proposed receptor binding site (Figure 6-5)[104]. Finally a third region of MIF, a partially buried cavity in the centre of the trimer, has an enzymatic tautomerase activity for a yet to be identified physiological substrate potentially containing an indole ring or ketone (Figure 6-5)[105-108].

While studies in this thesis suggest MIF binds an MHC class I binding receptor on NK cells, it is not implied that an MHC class I binding receptor mediates all biological functions of MIF. MIF weakly inhibited binding of a MHC class I tetramer to NK cells in ways that limited its endocytosis and enzymatic activities (Figure 4-6). Use of a version of MIF with a mutated thioredoxin motif will provide more confidence that the observed inhibition of MHC class I binding is due to a shared motif with MHC class I and not other functions of MIF that operate independent of endocytosis at 4 C.

MIF was originally identified as a lymphocyte secreted trimer that inhibited migration of macrophages, an affect that may also be attributed to induction of macrophage adhesion. Recently, the ability of MIF to activate Rho signaling, phosphorylation of focal adhesion kinase (FAK) and formation of stress fibres through myosin light chain kinase (MLCK) [103;109;110] was described, identifying putative pathways involved in increased adhesion and reduced migration of macrophages caused by MIF. Many of the effects of MIF in a spectrum of disease models may be explained by its ability to inhibit directed migration, or promote adhesion. The diminished pathology in many experimental diseases when MIF is eliminated [111] may be attributable to lessened chemotaxis and adhesion of immune cells that result in fewer destructive immune cells at affected tissues [103;112;113]. Adhesion and migration are also important components of angiogenesis, metastasis, apoptosis, and cell survival and may account for the effects of MIF in these processes [114-118]. Whether it is the receptor-mediated effects of MIF, or other MIF functions that inhibit macrophage adhesion or migration is not clear. Indirect effects of cell adhesion may cause many of the results attributed to MIF.

The several interactions and functions of MIF likely involve more than one region of MIF. A small region of homology to MHC class I in MIF could account for many receptor mediated functions of MIF. It is interesting, that one of the proposed receptors for MIF and MHC class I on NK cells, PIRB, affects cell migration and adhesion in a way much like MIF [119;120].

6.6.3. Cross-Reactive mAb: A Summary

In the course of this thesis research the binding of two mAb to proteins other than their recognized targets was described. In one case, the binding of anti-MHC class I mAb OX18 to MIF, the small region of similarity between MIF and MHC class I may be biologically relevant. The described effect of MIF on NK cell cytotoxicity [121;122], its localization in the body [123], and the location of the mAb epitope on MIF, all are consistent with a MHC class I-like function of MIF.

In the other case, without a precise definition of the epitope of anti-CD8α mAb D9 on CD8α or the F1 ATP synthase β subunit, it is difficult to predict if the small region of similarity between CD8α and the F1 ATP synthase β subunit is biologically relevant, as the two proteins, according to evidence currently available have no similar functions.

6.7. CD8: Re-examining the Co-receptor Concept

CD8 is often labeled as a TCR co-receptor. However what is signified by "co-receptor" varies widely across the literature. Sometimes "co-receptor" defines any receptor that enhances CTL activation (e.g.[124]), sometimes "co-receptor" appears to suggest CD8 binds, at an undefined point, the same MHC class I molecule that is later or earlier bound by TCR (e.g. [45]), and in other places "co-receptor" describes the ability of CD8 to bind the same MHC class I simultaneously with TCR (e.g. [125]). I will define CD8 co-receptor function according to the initial definition proposed by Janeway [125], wherein CD8 and TCR presumably bind the same MHC class I molecule, at the same time (Figure 6-6). To describe the ability of CD8 to promote T cell activation, as for example, integrins do as well, I will use the term "co-activation".

Must CD8 bind the same MHC class I as TCR to promote intracellular signaling and cellular responses? If CD8 can activate cells by binding MHC class I, without binding the same MHC class I molecule as TCR, it would assist our understanding of CD8 in stimulating monocytes, dendritic cells, or NK cells that do not express the TCR. Examination of the binding constants, orientation and contact sites of CD8 binding to MHC class I will help understand how CD8 promotes binding of TCR to MHC class I. With this groundwork, we may hypothesize a role for binding of CD8 to MHC class I in promoting binding of receptors other than TCR to their ligands or in directly initiating intracellular signaling.

6.7.1. The Evidence That CD8 Binds the Same MHC Class I Molecule as TCR is Indirect and May be Subject to Other Interpretations

The binding sites for TCR ($\alpha1\alpha2$ domains) and CD8 ($\alpha3$ domain) on MHC class I-peptide do not overlap (Figure 1-4)[51;52], suggesting TCR and CD8 may bind the same MHC-peptide complex. While proximal loops of MHC class I shift significantly when CD8 binds, these structural changes are not translated through MHC class I structure to the distant TCR binding site: nothing suggests preliminary binding of CD8 to the same MHC class I causes a conformational change in MHC class I that facilitates binding of TCR [22;126].

CD8 is often labeled a co-receptor for TCR, however the evidence supporting this not robust. The co-receptor name is often given CD8 due to a presumed ability to bind MHC class I at the same time as TCR. No crystal structure of a CD8-TCR-MHC class I complex, or evidence of a trimolecular complex of CD8-TCR and a single MHC class I that would conclusively demonstrate that CD8 and TCR bind MHC class I at the same time is available. Moreover, there is disagreement on whether the formation of a complex of MHC, CD8 and TCR is even possible without substantial structural distorsion [126;127]. While evidence, over more than 25 years, has accumulated to suggest CD8 can bind the same MHC class I as TCR, and is thus a TCR co-receptor, other interpretations are possible. Whatsmore, much evidence suggests binding of CD8 to a different MHC class I than TCR efficiently promotes T cell activation. I wish to carefully develop the evidence for and against a co-receptor activity for CD8, because if CD8 does not, cannot, or is not obliged to bind the same MHC class I as TCR to promote CTL activation, one can separate CD8 from its currently imagined restriction to co-activating TCR-initiated responses. If CD8 does not need to bind the same ligand as TCR to activate T cells, then by analogy CD8 may help activate monocytes, NK cells and dendritic cells through many receptors that bind MHC class I or other ligands in the absence of TCR.

CD8 binding to the same MHC class I as TCR is frequently represented in schema such as Figure 6-6. A review [125], or several early papers [128-130] are often cited to make this claim. However, this evidence is weak. These papers demonstrate that mutating the CD8 binding site of MHC class I can decrease or nullify T cell activation [130;131], even in the presence of wild-type MHC class I [128-130]. This has often been cited as evidence that CD8 must bind the same MHC class I as TCR to induce T cell activation [125]. However, supplying MHC class I that binds CD8 but doesn't activate TCR increases T cell responses [45;132-134]. As such, studies that suggest CD8 binds the same MHC class I as TCR [128-130] need to, but have not, quantitatively replaced TCR-activating-MHC class I-peptide that does not bind CD8 with equal amounts of MHC class I that binds CD8 but does not activate TCR. Indeed, where this was done there was minimal or no effect on T cell activation [45;132;134]. This suggests whether CD8 binds the same MHC class I as TCR makes little or no difference to T cell activation.

A photoaffinity system that cross-links antigen-specific MHC class I to TCR in the presence or absence of CD8-MHC class I interactions can quantitatively measure the amount that CD8 enhances binding of TCR to MHC class I [65;131]. However, it is not necessary to assume that CD8 binds the same MHC class I molecule as TCR, at the same time as TCR in these studies. The ability of CD8 to augment binding of TCR to MHC class I could be explained in at least two other ways: (1) CD8 may transiently interact with MHC class I slowing its movement in the plane of the plasma membrane in the required orientation to bind TCR, thus removing several degrees of freedom of MHC class I, and thereby enhancing the likelihood of TCR binding MHC class I after its release from CD8; (2) Linking of CD8 and TCR through intracellular complexes containing lck, LAT, CD3ζ and ZAP-70, the CD8 stalk region and transmembrane region may further encourage CD8 function described in (1).

6.7.2. The Case for Co-Receptor Activity of CD4

Caution must be used in extrapolating findings with CD4 to CD8 as structurally the two "co-receptors" are quite dissimilar, CD4 being a monomer with four extended Ig domains, and CD8 being a dimer, each unit of which has one Ig domain. Thus, CD4 and CD8 may not use all the same strategies to promote T cell activation. That said, Mark Davis' lab has offered perhaps the strongest evidence that maximal activation of CD4+ T cells requires that CD4 bind the same MHC class II as TCR [135]. Dimers of two different MHC class II molecules were used to quantitatively compare the effect on T cell activation of CD4 binding to the same or a different MHC class II molecule as TCR. CD4 increased T cell sensitivity about 100-fold if it bound the same MHC class II as TCR [135]. Again, CD4 and TCR may not simultaneously contact the same MHC class II molecule. The assays used were not titrated through the full range (10^6) of CD4 or CD8 effect on T cell activation, and thus only minimally visualized stimulation of T cells due to CD4 binding to a different MHC class II than TCR [135]. While similar studies have been performed on CD8, the necessary controls and iterations of the experiments were not performed, to suggest that CD8 must bind the same MHC class I molecule as TCR to most efficiently promote CTL activation [134]. Mark Davis' study with MHC class II dimers echoed earlier studies that suggested that CD8 can co-activate T cells with TCR whether or not it binds to the same MHC class I. If CD8 can bind the same MHC class I as TCR, T cells are more efficiently activated. In these studies if TCR was specific for MHC class II, CD8 multiplied T cell responses by up to 9 fold, whereas if TCR bound MHC class I, like CD8, T cell responses were increased 10-100 fold [125].

6.7.3. CD8 Binds MHC Class I Before TCR On Average Due to its Faster Binding Association Rate

CD8 may aid TCR in scanning the surface of target cells or antigen-presenting cells in search of specific MHC-peptide complexes, which will activate signaling through TCR. TCR binds MHC-peptide with a high affinity (0.1-10 μM), but a very slow association rate (10^3-10^5/ Ms^{-1}, typical of induced fit binding [126]. CD8 has a lower binding affinity for MHC class I than TCR (50- <200 μM) but a faster association rate typical of rigid body binding (1.4 x 10^5/ Ms^{-1}) [136]. Therefore, CD8 will on average engage MHC before TCR binds a low affinity/weak agonist MHC class I-peptide, when CTL contact a potential target cell. Moreover, MHC class I may have a supine orientation on the plasma membrane, with the CD8 binding site most overtly exposed to the extracellular space and the TCR binding site partially hindered by the membrane [137]. If this is the normal orientation of MHC class I on the membrane, CD8 may establish first interactions with MHC class I and TCR may be obliged to bend to engage MHC class I. Thus, CD8 may stabilize contact regions between CTL and target cells at an appropriate intercellular distance for TCR-MHC-peptide recognition, and reduce the three-dimensional randomness in TCR-MHC binding, augmenting TCR binding to specific MHC-peptide. Experimental evidence supports this possibility. Binding of antigen-specific MHC class I tetramers to T cells is enhanced approximately 2-fold by CD8 [20;21;138;139], and CD8 increases the amount of MHC class I bound to TCR at a given time [131]. Nonetheless, CD8 does not affect the TCR-MHC class I disassociation rate, in line with the fast off-rate of CD8 compared to TCR [21;140]. Thus CD8 aids initial binding of MHC class I to TCR but does not affect disassociation of MHC class I from TCR. In agreement, binding of MHC tetramer to CTL is biphasic [141]. The early phase of binding can be blocked by anti-CD8 mAb, has binding rate constants that are not affected by different clones (TCR-affinity independent), and are of similar affinity to CD8 [140;141]. CD8 likely accounts for the first stage of MHC class I binding to T cells, while the TCR accounts for later MHC class I binding [140;141]. This evidence suggests CD8 facilitates TCR scanning of endogenous MHC class I peptide complexes for antigen-specific peptides, promoting initial TCR detection of MHC class I, and enhancing sensitivity of target cell recognition[142]. TCR specificity appears to depend on the TCR disassociation rate, or TCR-dwell time on MHC class I [143], as a measure of binding affinity for TCR-activating peptides loaded on MHC class I. Thus, because CD8 does not affect TCR disassociation rate, it should not affect TCR specificity [21;140;142]. This model could account for the ability of CD8 to enhance binding of TCR to MHC class I, without CD8 and TCR being obligated to bind the same MHC class I.

6.7.4. *Fast On-Rate Binding of CD8 May Promote Binding of Slow On-Rate Receptors to Their Ligands*

The fast on-rate rigid body binding of CD8 combined with the slow on-rate induced fit binding of TCR [144] may allow CD8 to enhance binding of MHC class I to TCR. If a monocyte, dendritic cell, or NK cell receptor, like TCR, had a slow-on-rate of binding to MHC class I and preferably a binding site on MHC class I that does not overlap with CD8, CD8 may be able to enhance its ability to bind MHC class I and responses it elicits. KIR [145;146] and ILT2 and 4 [147;148] have affinities for MHC class I that are similar to TCR (Table 6-2), but they bind with fast-on rates, like CD8, and therefore CD8 would probably be of small benefit to their recognition of MHC class I ligands (Table 6-2). In contrast some Ly49 family members have slow on-rate binding kinetics for MHC class I like TCR (Table 6-2) [25]. CD8 may promote binding of receptors with uncharacterized binding affinities, or some lectin receptors to MHC class I as it does for TCR. This may allow CD8 to promote immune responses directed at MHC class I expressing targets through receptors other than TCR.

Alternatively, if CD8 does not need to bind the same MHC class I molecule at the same time as TCR to enhance TCR-MHC class I binding, CD8 may enhance binding of many receptors with TCR-like kinetics no matter what their ligands. In this model CD8 would bind MHC class I on a target cell, thereby optimizing intercellular distance and membrane orientation for binding of other receptors with induced-fit kinetics. In so doing, CD8 may promote binding of lectins, FcR, Ig-like receptors or others to their ligands on target cells and promote immune responses through several receptors.

6.7.5. Alternate Interpretations of Data Demonstrating CD8 Promotes Binding of TCR to Antigen-Specific MHC Class I

Taken together, current data demonstrates CD8 enhances the binding of TCR to MHC class I-peptide, in turn enhancing T cell responses. Indirect evidence suggests that CD8 can bind the same MHC class I-peptide as TCR, but other explanations are possible. For example, interactions of lck, LAT, ZAP-70, CD3ζ and formation of optimal signaling complexes may require a particular extracellular orientation of CD8 and TCR, involving binding to the same MHC class I concurrently, even if this is secondary to formation of intracellular signaling complexes or other indirect interactions of CD8 and TCR (Figure 6-7). CD8 may associate with TCR in several direct and indirect manners, not involving MHC class I, to promote TCR binding to MHC class I. The stalk, transmembrane and cytoplasmic regions of CD8β promote CD8 association with TCR and may further enhance binding of TCR to MHC class I [65;149]. It is interesting that when surface plasmon resonance (SPR) was used to measure the influence of CD8 on TCR binding to MHC class I, the one study utilizing recombinant CD8 including the stalk region found that CD8 increased MHC class I binding to TCR [150], while no effect was found when the stalk region was excluded [65;151]. However even if CD8 does not bind the same MHC class I-peptide as TCR it still can co-activate by several-fold T cell responses. This suggests CD8 is not restricted to co-receptor function, and is therefore not restricted to activating responses only through TCR. CD8 may operate with other receptors besides TCR (Table 1-3).

6.7.6. CD8 as a Co-Stimulatory Molecule That Relies on Initial Priming of TCR

Conserved regions of MHC class I that are widely expressed are often bound by receptors containing immune receptor tyrosine based inhibitory motifs (ITIM) that inhibit responses of

T cells, NK cells, macrophages, dendritic cells and mast cells. According to the original theory of Karre this is to prevent immune responses against "self" [152]. Within this theory CD8 is somewhat unique in binding a conserved region of a ubiquitous protein (MHC class I) and being an activating receptor. While a role for CD8 alone in regulating cell survival has been proposed this effect may rely only on TCR and be a result of T cell expansion in lymphopenic mice that does not occur under normal physiological conditions [153]. As nearly every cell expresses MHC class I with the potential to bind CD8, it is crucial, in theory, that cytotoxicity activated through CD8 be carefully regulated. Part of the regulation of CD8 activation may be mediated by changes in the level of MHC class I expressed in inflammatory environments or certain tissues. Activation through CD8 may also be regulated by co-expressed ITIM containing inhibitory receptors, or by competition for binding to MHC class I with other receptors like immunoglobulin-like transcript (ILT)-2 [147]. However on T cells, control of CD8 activation may be exerted through CD8-dependence on co-activation through TCR. CD8 on cells other than T cells may be similarly regulated via a requirement for co-activation through a receptor specific for a pathogen or activator [e.g. Receptor for Fc of immunoglobulin (FcR), Toll-like receptor (TLR), NKG2D (NK cell receptor D), ILT]. One might postulate that as CD8+ monocytes, macrophages, dendritic cells and NK cells respond to both cellular and non-cellular stimuli (e.g. opsonized bacteria or cancerous cells through FcR), CD8 by binding its ubiquitous ligands associated with the cellular stimuli, may differentiate responses to cellular stimuli from responses to non-cellular stimuli. This could promote tailored responses of CD8+ innate immune cells to the context of stimuli.

A significant body of work has examined the ability of CD8 to activate TCR-initiated responses as an accessory adhesion and co-stimulatory protein, independent of any co-receptor function in TCR binding to antigen-specific MHC class I (Figure 6-7) [77;124;132;133;154-156]. In these studies, initial priming of TCR enhances binding affinity or avidity of CD8 for MHC class I. Binding of CD8 to MHC class I after TCR-priming resulted in Ca+2 fluxes and phosphoinositide signaling [124;156]. These CD8-mediated effects are consistent with recruitment of PLCγ and PI3K after phosphorylation of LAT[157;158] (potentially associated with CD8[66]). Thus, CD8 may function as an accessory, co-stimulatory molecule at least in part by recruiting, or being recruited by LAT.

Preliminary stimulation through TCR, below the threshold that initiates CTL degranulation is essential to enable these CD8 co-stimulatory functions. As discussed previously and below, TCR shares identical or interchangeable signaling mechanisms with other immune system receptors. Some of these receptors include FcγRIII on NK cells, FcγRI or γIII on monocytes, or ILT1 (see below). A preliminary sub-threshold stimulation through one of these receptors may activate CD8 accessory functions, enhancing its binding to MHC class I and instigating Ca+2 fluxes and phosphoinositide signaling. Through accessory functions, CD8 may enhance the responses of several receptors on monocytes and macrophages, without co-receptor function.

6.7.7. Speeds and Pauses: An Alternate Theory of the Role of CD8 in Cell Activation

While it was thought that extracellular interactions of receptors led to specific changes in intracellular conformation that elicited cellular activation, this is now thought to be the exception (e.g. CD3 [159], affinity enhancement of integrins [160],) rather than the rule. In fact, while the interactions of CD8 and TCR with MHC class I are weak [151], they may be multiplied by transient intracellular interactions (e.g. lck with CD3ζ) and stabilized by formation of LAT-based scaffolds with a minimum 1000-fold stronger affinity [161]. This suggests a model as proposed recently by Alain Trautmann [162] wherein random and rapid movements of kinases, adaptors, and receptors are independently slowed enough by interactions, even of low affinity (for example of CD8 or TCR with MHC class I), that the cumulative result is microclusters of activated TCR, CD8, kinases and adaptors (Figure 6-8). These microcluster aggregates given a threshold of "slowing" ligands, would then form aggregates of aggregates and eventually reach a necessary threshold or quorum to activate T cells (Figure 6-8). Thus extracellular detection and intracellular responses both positively feedback on each other and translate their respective environments through the plasma membrane in terms of 3-D trajectories, speeds, pauses and locales rather than static single protein conformational changes.

The model [162] is based on evidence that tens of molecules, including lck, ZAP-70 and TCR form "microclusters" that appear in tandem with T cell activation [163;164], and fits with evidence that compartments of the plasma membrane are the size of a few proteins, not vast complexes [165]. The model accounts for many known variants of T cell activation. CD8-dependent and independent T cell activation can be accounted for by different TCR dwell times resulting in varying degrees of slowing, pauses and aggregations. The model allows that each domain of CD8α and CD8β may contribute to T cell activation through their independent abilities to promote interaction with TCR, MHC class I, lck or lipid rafts, as has been observed [65;149;166]. Finally, it suggests the ability of intracellular components to recruit TCR and CD8 to the same site, independent of their binding to MHC class I. Several lines of evidence support this. CD8 or CD4 and TCR do not co-aggregate through binding to MHC [19;167-169], but through intracellular interaction of lck with the TCR-CD3-ZAP-70 complex [170;171]. Furthermore, lck association of CD8 may contribute to the ability of CD8 to enhance TCR-MHC class I binding [65]. This model suggests CD8 will be recruited to the site of TCR activation and potentiate T cell responses through multiple interactions, both intracellular (lck, LAT) and extracellular (binding MHC class I, binding TCR). Acknowledging that multiple short-lived contacts foster T cell activation this model obviates the need to rely on a model wherein CD8 binds the same MHC class I as TCR at the same time. Furthermore, at different stages of T cell development, or on different cell types, CD8 may rely more predominantly on, or use exclusively only some of its means to promote T cell activation (e.g. lck, LAT, binding MHC class I, binding TCR, associating with lipid rafts).

6.8. CD8α on Human Monocytes Co-Activates FcγR-Dependent Responses: A New Paradigm in CD8 Co-Activation With Receptors Other Than TCR?

While CD8 on T cells influences cell activation toward survival, proliferation, cytotoxicity, apoptosis, or rapid cell death after its co-ligation with TCR comparatively few studies have described a function of CD8 on other cell types. This probably results from the uncertainty concerning how CD8 may function when it cannot rely on co-stimulation through TCR. Some studies described roles for CD8 in survival, apoptosis and cytokine production by NK cells and macrophages and suggested these effects were dependent on ZAP-70/Syk kinase and src kinases, much like TCR signaling. However, a model had not been explicitly presented that would cohesively describe whether CD8 acts independently or in concert with other receptors, how constitutive activation of CD8 by near-ubiquitous MHC class I would be prevented, and particularly how CD8 would signal on monocytes, macrophages and dendritic cells that lack lck, a key CD8-signaling mechanism. Some experiments in this thesis have begun to test the basic tenets of the model, presented in the introduction, that CD8 may co-activate responses of receptors, like FcR, that use similar, but not identical signaling mechanisms to TCR.

6.8.1. CD8α on Monocytes Enhances Signaling and Downstream Responses with FcγR

An advance made in these studies is evidence suggesting that CD8 on human monocytes enhances signaling through receptors other than TCR (Figure 6-9). Ligation of CD8 in conjunction with stimulation with immune-complexes enhanced human monocyte release of TNF in a CD8-dependent manner. TNF production could be blocked by excess soluble Fc fragment of Ig, suggesting the responses were Fc-dependent and likely mediated by FcγR. Furthermore, as predicted by the model of CD8 signaling through γ-chain linked receptors, immune-complexes induced phosphorylation of LAT, and phosphorylation was enhanced by ligation of CD8 (Figure 6-9).

It is possible that the inhibition of monocyte TNF release with Fc fragments could be due to ligation of inhibitory FcγRIIb receptors by Fc fragments, rather than competition with immune-complexes for binding to activating FcγR. Which FcγR(s) are involved in these CD8-enhanced monocyte responses is not clear, as all signal through Syk and at least FcγRI and FcγRIIa signal through LAT [172-174], and may therefore have their responses amplified by CD8 and blocked by excess Fc. The model suggests CD8 would preferentially enhance responses of FcγRI and FcγRIII because they associate with and signal via the common γ chain, rather than FcγRIIa that signals through an internal ITAM rather than via γ chain ITAM [175]. Mouse IgG_{2a} mAb, that bind with a high affinity to FcγRI [176-178], were used to activate monocytes through FcγR. While these data suggest that responses of FcγRI are most likely to be enhanced by CD8 (associates with common γ chain, binds mouse IgG_{2a}, and LAT) study of each FcγR, either with specific mAb, siRNA knockdown, or a reconstituting transfection system will be required to demonstrate which FcγR(s) have their responses enhanced by CD8.

The model of CD8 function with receptors other than TCR (Figure 1-6) predicts that simultaneous stimulation through CD8 and FcεRI would amplify mast cell responses, as both FcεRI and other FcγR on mast cells signal through common γ chain, Syk and LAT [179;180]. Unfortunately the effect of CD8 on mast cell FcεRI responses was only measured 48 hours after treatment with anti-CD8α mAb [26]. After 48 hours the majority of anti-CD8α mAb was probably internalized and/or degraded, and would have no ability to co-activate signaling through CD8 and FcεRI. This could explain why anti-CD8α did not change mast cell phenotype after 48 hours in a way that enhanced mast cell activation [26].

In studies with human monocytes (this thesis) anti-CD8α mAb was aggregated with anti-Ig antibodies to induce monocyte TNF production and CD8α appeared to depend on FcR stimulation to activate monocytes (Figure 2-7). Additionally, several anti-CD8α mAb did not stimulate monocyte TNF production in monomeric form (Figure 2-7). In contrast, monomeric anti-CD8 mAb induced rat macrophage and mast cell responses without the need for additional antibody to cross-link and cluster anti-CD8 mAb [18;26;88]. It is possible rat and human CD8 differ in their dependence on co-activation or signaling abilities. Another possibility is that a small proportion of aggregated anti-rat CD8α mAb in commercial preparations of monomeric anti-CD8α mAb induced co-activation of FcγR and CD8 on rat macrophages and mast cells in the same way as the anti-CD8α mAb aggregates purposefully used in studies of human monocytes. Finally, the Fc portion of monomeric anti-CD8α mAb may have co-ligated FcR and CD8 on rat cells, allowing CD8 enhancement of FcR-dependent responses. No evidence is available to suggest the FcγR-independence of rat macrophage and mast cell stimulation through CD8 that used F(ab)$_2$ fragments of anti-CD8 mAb or other methods.

In studies of NK cells and γδ T cells that have demonstrated CD8-dependent cytokine release or effects on apoptosis and survival [181] the possibility that cellular responses are due to co-stimulation of CD8α and another receptor cannot be excluded. As outlined above anti-CD8α mAb may co-stimulate CD8α and FcγR on NK cells or γδ T cells. Likewise, in studies that have used MHC class I ligands to elicit CD8-dependent effects, the co-stimulation of cells through CD8 and a second receptor for MHC class I cannot be excluded [182-185]. The ability of CD8 on T cells to affect T cell cytotoxicity appears to depend on stimulation through TCR. Similarly, the few published studies demonstrating a role for CD8 on cells without the TCR allow the possibility that CD8 on macrophages, mast cells, NK cells, and γδ T cells can elicit cellular responses only when co-stimulated through a second receptor such as FcγR.

Further definition of the role of FcR and LAT in CD8 enhancement of monocyte and macrophage responses may bolster a wider appreciation of the ways CD8 functions on cells other than T cells.

6.8.2. An Accessory to Monocyte Activation: Is CD8 Co-Activation of Monocyte Responses Dependent on Priming Through FcR?

CD8 promotes binding of TCR to MHC class I. After initial TCR activation, P13K-sensitive cytoskeletal re-arrangement results in "activated" or enhanced adhesion of CD8 to MHC class I through clustering of CD8 or other avidity effects [77]. Increased adhesion of CD8 to MHC class I after TCR priming may in turn enhance intracellular signaling through

CD8 as Ca^{+2} flux and phosphoinositide signaling are activated in a CD8-dependent manner [124;133;156]. Enhanced binding and signaling of CD8 after TCR priming has been called CD8 accessory function. CD8 on T cells thus has at least two roles (1) initial promotion of TCR binding to MHC class I, and (2) CD8 accessory function (TCR-activated binding of CD8 to MHC class I and intracellular signaling). In contrast, CD8 on monocytes may not promote binding of monocyte receptors to their ligands and may only exhibit CD8 accessory function. Thus, initial priming of monocytes through FcR, or other receptors may enhance CD8 adhesion to MHC class I, and intracellular signaling resulting in Ca^{+2} flux and phosphoinositide signaling.

The first suggestion of activated CD8 accessory function on monocytes is provided in this thesis. Adhesion of T cells to MHC class I is enhanced after activation through TCR, and this adhesion can be inhibited with anti-CD8 mAb and partly depends on CD8-binding sites on MHC class I [44;133]. Incubation of rat macrophages with mouse IgG_{2a} increased their adhesion to MHC class I, and increased adhesion was inhibited with anti-CD8 mAb (Figure 2-6). This hints that CD8 may participate in signaling initiated through FcR but requires confirmation. As immune cell receptors often use overlapping or similar downstream signaling mechanisms (e.g. activation of P13K), it is perhaps not surprising that activation of cells through either FcγR or αβ TCR may enhance adhesion of cells through CD8 to MHC class I. As in T cells [45], initial signaling through some macrophage receptors, like FcγR, may amplify subsequent adhesion and/or cytotoxic responses by enhancing CD8 adhesion to MHC class I.

CD8 accessory function may be activated by several receptors like FcγR, to initiate CD8 binding to MHC class I and intracellular signaling. In the temporal scheme of TCR signaling lck is involved in initial TCR activation and LAT is recruited downstream. Thus, early TCR signaling may be enhanced by CD8 bound to lck and as CD8 accessory function occurs downstream of initial TCR activation it may predominantly involve LAT. Monocytes are not believed to express lck [186], and therefore CD8 on monocytes would not be expected to bind or activate lck signaling. Therefore, CD8 may be involved not in early, but intermediate signaling of monocyte receptors in a role resembling CD8 accessory function on T cells. The intracellular signaling associated with CD8 accessory function after TCR priming (Ca^{+2} flux, phosphoinositide signaling) is consistent with recruitment of PLCγ and P13K by phosphorylated LAT [124;156-158]. In studies here, CD8 enhanced phosphorylation of LAT on a tyrosine important for recruitment of P13K and PLCγ [157;158]. Thus, this thesis shows that Ig enhanced CD8-dependent binding to MHC class I, and immune-complexes enhanced signaling through LAT, consistent with an accessory function of CD8, activated secondary to FcγR stimulation.

CD8 on monocytes and other cells without the TCR may contribute to cell activation through CD8 accessory function alone. If this is the case, the impact of CD8 on activation of receptors other than TCR may be less dramatic than its exponential enhancement of some TCR-mediated responses. However, depending on precisely how CD8 accessory function is activated, CD8 may enhance the responses of several monocyte, macrophage and dendritic cell receptors. For example, if CD8 accessory function is activated by phosphorylation and recruitment of LAT (attached to CD8) to signaling complexes at site of TCR or FcγR ligation, this could result in CD8 clustering and enhanced avidity for MHC class I. In this case, CD8 would exhibit accessory enhancement of any receptor that recruits LAT including: FcγR

[172;174], FcεRI on mast cells [179], ILT1 (and potentially other ILT family receptors) [172;187], 2B4 [188], CD2 [189], and gpVI [190] (Table 1-3).

6.9. Primordial Functions of CD8: TCR Co-receptor or MHC Class I Binding/Signaling Protein

In mammals CD8 is expressed by several cell lineages that do not express TCR. Hence, one might ask if the primordial function of CD8 was as a TCR co-receptor, a TCR co-activator, or an adhesion and signaling protein involved in innate immunity. Consideration of innate immune cell types that express CD8, and the signaling mechanisms CD8 likely used in primordial species may provide novel insight into functions of CD8 on monocytes, dendritic cells and NK cells.

Jawless fish such as lamprey, other non-vertebrates and plants[191] have adaptive immune systems unrelated to systems of mammalian adaptive immunity like TCR, but no CD8 homologs are detected in these species [192] [192;193]. CD8 is first detected in jawed fish and later vertebrates [194-196]. Jawed fish, along with CD8, saw the simultaneous emergence of MHC class I, NK cells and the mammalian-type adaptive TCR system, in a "big bang" of rapid evolutionary development in the immune system[191;192;195;197]. Due to the simultaneous and rapid evolutionary development of MHC class I, NK cells, CD8 and the TCR it will likely be difficult to judge whether CD8 emerged as a MHC class I binding receptor or a TCR co-receptor.

T cells and NK cells share parts of their profile of expressed proteins; both can express several MHC class I receptors. It may be possible CD8 evolved as a MHC class I binding receptor expressed by both T cells and NK cells, if CD8 is expressed by both NK cells and T cells in fish. Unfortunately, at this juncture fish T cells and NK cells have not been separated for analysis of CD8α expression either at rest or when CD8α is robustly upregulated after alloantigen stimulation [194]. At a low number of RT-PCR cycles CD8α is not detected in a mixture of fish monocytes and neutrophils [194]. While low levels of CD8 expression on fish monocytes and macrophages may have escaped the low sensitivity analysis used, this suggests ancestral forms of CD8 may have emerged on TCR+ cells or NK cells, and not monocytes [194].

6.9.1. CD8α Binding Loop of MHC Class I is Conserved in Fish, Amphibians and Reptiles

Additional detailed analysis of sequence and functional conservation of CD8, TCR and MHC class I in evolution may give further insight into whether CD8 emerged as an MHC class I binding receptor or a TCR co-receptor. Charged residues composing the CD8-binding loop of MHC class I are conserved in fish, reptiles, amphibians, birds and mammals [198;199], suggesting that CD8 from its emergence had an important MHC class I binding function. In contrast, some evidence suggests CD8 in fish lacks several functions thought to be important for its co-receptor function with TCR. The evidence that suggests CD8 may have emerged as an MHC class I binding receptor, independent of T cell-specific functions with the TCR is outlined below.

6.9.2. Fish CD8α Lacks the Lck Binding Site

CD8α may have evolutionarily originated from a function independent of TCR, and even lymphocytes. As discussed above, Syk replacement of ZAP-70 obviates the need for lck [200]. Thus if CD8 functions on monocytes or NK cells with receptors that signal through Syk rather than ZAP-70, CD8 may signal through LAT, and lck may not required or expressed. CD8α in all the species of fish examined lacks the consensus motif for binding src kinase lck [197;201], despite fish CD4 retaining the consensus site for binding lck [202]. This suggests CD8 may function in some species independently of the seemingly lymphocyte-restricted src kinase lck [203]. Given that LAT is expressed in the monocytic THP-1 cell line, mast cells and other non-T cells that express CD8 [172;204], it is possible CD8 emerged first as an MHC class I binding, LAT-associated receptor without restriction to TCR or lymphocytes. Secondarily CD8 may have become fine-tuned to bind lck and function with the TCR, partly via gene duplication that generated CD8β [43].

6.9.3. CD3ζ and Common γ Chain Derive From a Common Ancestor

While it is not known if homologues of LAT exist in fish, LAT can operate in mammals with both adaptive and innate immunity receptors. Consequently, if CD8 binds and signals via LAT, CD8 may have an evolutionary origin not restricted to TCR signaling. LAT is phosphorylated by ZAP-70 or Syk kinase [205] which are recruited to phosphorylated CD3ζ or common γ chain. Common γ chain and CD3ζ were generated via ancestral gene duplication [206;207], and common γ chain can substitute for CD3ζ in several situations including FcγRIII (CD16) function [208] and T cell activation through TCR [209], suggesting common γ chain operates adequately with TCR signaling pathways like lck and LAT (also see section "On Myeloid Cells CD8α May Signal Via Common γ chain, Syk and LAT"). Thus, CD8 signaling pathways may have co-evolved with common γ chain and innate immune receptors, not just the TCR-signaling CD3ζ chain.

6.9.4. Predecessors of the TCR on NK Cells and Monocytes

Several receptors on innate immune cells are thought to be evolutionary precursors of TCR due to sharing of the relatively rare V-C1-type Ig domain [195;210]. CD8 may have functioned in early evolution and perhaps still functions in some mammalian species with these innate immunity receptors. Some of the receptors proposed to be ancestors of TCR include SIRPβ which is expressed by monocytes and retains a signaling mechanism homologous to TCR [211;212], T cell/NK cell receptor CRTAM (that partially conserves the TCR α-cpm sequence important for association with CD8 [see appendix 2]), and widely expressed nectin family adhesion receptors (Table 1-3)[195;210].

CD8β may associate with CD3δ as a means of promoting CD8 co-operation with the TCR [213]. While CD8β is conserved from fish to mammals [196], CD3δ is a gene duplicate of a primordial CD3δ/γ gene found in chickens and lower animals [214]. This suggests that while interaction of CD8β with CD3δ may optimize CD8 function with TCR in mice, it may not be conserved in evolution and may not be essential for CD8 co-activation of TCR-initiated responses. On the other hand, CD3δ has been found in cells other than T cells such as human monocytes and macrophages, B cell lymphoma cells, bone marrow progenitor cells and mouse CD8+ dendritic cells (see below "Common Traits: CD8 as Part of a Cytotoxic Expression Profile in Multiple Cell Types") suggesting association of CD3δ and CD8 may promote activation of receptors other than TCR.

From an evolutionary analysis, it seems similarly probable that CD8 emerged as a TCR co-receptor or an innate immunity receptor with the ability to bind MHC class I and signal. CD8-dependent signaling mechanisms exhibited a parallel development in innate and adaptive immune cells. CD8 in lower vertebrates probably binds MHC class I, but may not bind lck, which is thought to be important for CD8 co-activation of TCR-initiated responses. Taken together, CD8 may have had from the beginning of its evolution a broader mandate than TCR and lymphocyte specific functions. Primordial innate immune functions of CD8 may be observable in present-day mammals.

6.10. Common Traits: CD8 as Part of a Cytotoxic Expression Profile in Multiple Cell Types

Expression of CD8α is not controlled by elements unique to the CD8 promoter. CD8 expression is controlled by large-scale chromatin changes that generally regulate large groups of genes (reviewed in [68]). As these large-scale chromatin changes are regulated processes they often control expression of a spectrum of genes involved in many levels of a biological process. Therefore, CD8 may be co-expressed with many proteins involved in the same biological processes including TCR signaling or cytotoxicity (Table 6-3).

Transcriptional programs assigned to distinct immune cell lineages, such as myeloid and lymphoid cells, may be somewhat fluid. It seems reasonable that transcriptional programs would be oriented towards particular functions, rather than particular cell types. For example, if a myeloid and lymphoid cell are both capable of cytotoxicity, it would be logical that they use shared or overlapping proteins and transcriptional programs to enable this ability. This appears to be the case.

When CD8 is expressed by dendritic cells, monocytes, or even a B cell mantle zone lymphoma, other genes associated with CD8+ CTL are often co-expressed (Table 6-3). CD8 is expressed in a small percentage of B cell chronic lymphocytic leukemia (B-CLL) [215;216]. Mantle zone B cell lymphoma co-expressed perforin, granzyme B and CD8β with CD8α (Table 6-3) [217]. Like CD8, ZAP-70 is often [218;219] and lck [215] and CD3 [220] are sometimes expressed in B-CLL. Mouse CD8+ dendritic cells co-expressed CD3δ, CD3ε, and pre-TCRα, had IgH D-J rearrangements, and even expressed TCRβ paired with a novel 33 kDa protein[221] (Table 6-3)[222-226]. Mouse CD8+ dendritic cells may be cytotoxic [223].[225;227] Expression of CD8 and CD3 also correlates on human NK cells: CD3+ NK cells are usually CD8+ [228]. Most interestingly for this thesis, rat CD8+ monocytes co-expressed perforin, granzyme B, and NKR-P2 a homolog of NKG2D, and NKRP1a at higher levels and caused more cytotoxicity than CD8- monocytes [31] (Table 6-3)[30;229]. It is possible low levels of the proteins involved in cytotoxicity and signaling with CD8 were not detected in monocytes, macrophages, dendritic cells and NK cells by the less sensitive techniques in use when these signaling proteins were decided to be lymphocyte-specific 20-odd years ago. In sum, the evidence suggests that control of CD8 expression may occur within a wider program of gene expression that regulates a particular function (e.g. cytotoxicity), phenotype (CD3 expression) or cell lineage (Table 6-3).

Ikaros and SWI control CD8 expression [16;230] and preceded the development of T and B cells in jawless vertebrates [231]. In jawless vertebrates and mammals, Ikaros expression is not T cell specific. Ikaros is expressed in human monocytes [17;232] and dendritic cells [233], where it upregulates lck, granzyme B, and FLT-3 receptor expression [234-236]. FLT-3 in turn upregulates other T cell associated proteins like CD3δ, ε, and ξ [237]. FLT-3 also causes *in vitro* expansion of CD8+/TCRαβ-/TCRγδ- plasmacytoid cells [222]. Ikaros has at least seven spliced forms [236], that may allow expression of CD8, lck, granzyme B and other genes formerly thought to be lymphocyte-specific in monocytes and dendritic cells. Thus, Ikaros and FLT-3 may be two key molecules used to activate a wide transcription program including multiple genes involved in T cell signaling and cytotoxicity, and including CD8, granzyme, perforin, and CD3 proteins. Expression of Ikaros or treatment with FLT-3 may induce CD8 and cytotoxic functions in monocytes and dendritic cells. It will be interesting to assess with this broad vision of CD8 function and contemporary sensitive techniques what epigenetic, transcriptional, and functional factors may be shared between CD8+ T cells and CD8+ myeloid cells, that may enable them to exert similar effects, such as cytotoxicity.

6.11. Effects of CD8 on NK Cells, Monocytes and Dendritic Cells at the Level of the Cell and Animal

In T cells activation of CD8 with TCR may result in T cell apoptosis, survival or CTL mediated cytotoxicity due to variation in strength or quality of signaling. Significantly less is known about the functions of CD8 on NK cells, monocytes and dendritic cells than is known about CD8 on T cells. However, in the past 10 years the presence of CD8+ monocytes and macrophages at sites of inflammatory disease has been identified, and the function of CD8 on these cells can be sketched. Much like CD8 on T cells, CD8 on monocytes, NK cells and dendritic cells may be involved in mediating cellular survival, apoptosis and cytotoxicity.

6.11.1. Modulating Signaling Through CD8 can Induce Survival or Cell Death

CD8 appears to be involved in signaling NK cell responses similar to T cell responses, such as survival or FasL induction. One laboratory has shown that binding of soluble MHC class I to CD8 or mAb cross-linking of CD8 induced FasL release and Fas-mediated apoptosis of NK cells, or T cells [182;183;238;239]. In contrast, much evidence suggests ligation of CD8 can induce a survival signal, or suppress an apoptotic signal in T cells or NK cells. As examples, mutation of the CD8α binding site in MHC class I tetramers induces Fas apoptosis of T cells [240;241]. In another study, while apoptosis was rapidly induced after NK cells bound target cells, the CD8+ NK cell subpopulation survived, and survival appeared to depend on CD8 and MHC class I [181].

Differences in the valency/avidity of CD8/TCR ligation may account for generation of apoptotic versus survival signals: monomeric MHC class I induced FasL production and apoptosis [182],[183;238;239] whereas tetrameric or cell-surface MHC class I allowed survival [181;240;241]. Similar signaling mechanisms of CD8 on T cells and NK cells may induce similar downstream signals, the variation of which can alternately result in survival or apoptosis.

6.11.2. Elimination of Antigen-Specific T Cells Via CD8 Expressed By Several Cell Types

CD8+ cells have long been recognized as "veto cells" that can eliminate CTL in an antigen-specific manner with outcomes such as the avoidance of graft-versus-host-disease and successful engraftment of transplants. Several types of CD8+ cells is capable of a veto function including CTL, activated NK cells, and activated T cells [242]. Within this heterogeneity, the veto affect is mediated in most circumstances via induction of FasL and Fas-mediated killing [243-245], or production of TGFβ [246;247].

A significant amount of evidence suggests CD8 on macrophages or DC may be directly involved in a veto-like effect. While one study found that a lack of CD8 on splenic CD8+-type DC from CD8 null mice had no implications for veto cell-like functions [248], similar experiments using blood or bone marrow DC, or T cells demonstrated an essential role for CD8 in a veto-like effect [223;245]. It is possible blood and bone marrow DC but not splenic DC exert a veto effect.

Whether CD8 is directly involved in the veto effect or is indirectly involved in development of veto cells cannot be distinguished by studies with CD8 null mice (e.g.[222;223]). Studies that demonstrate ligation of CD8α on a variety of cells induces FasL or TGFβ production suggest that *in vivo*, ligation of CD8 may induce a veto effect. Monoclonal antibody ligation of CD8 on undefined veto cells induced production of TGFβ [246;247]. As discussed above, many studies suggest that varying the level of stimulation of T cell or NK cell signaling via CD8 induces FasL expression and Fas-mediated apoptosis [182;183;241;249;250]. Thus, activation through CD8 on several cell types, even without TCR, may be responsible for FasL or TGFβ-dependent veto effects.

There was no antigen-specificity to the Fas-mediated apoptosis induced upon ligation of CD8 on T and NK cells *in vitro*. *In vivo* antigen-specificity may be provided to the veto effect by the induction of Fas expression after T cell activation [251]. As T cells specific for a transplant, for example, will account for most activated T cells, parallel CD8-mediated induction of FasL on a veto cell may selectively eliminate activated, and thus antigen-specific, or transplant-specific T cells.

A thorough understanding of the role of CD8 in the veto effect and activation of T cells could lead to therapies for transplantation, autoimmune diseases like diabetes, and viral infections.

6.11.3. A Potential Role for CD8 on Monocytes in Regulating Survival, Apoptosis and the Veto Effect

If CD8 participates in signaling through common γ chain-linked receptors, several roles for CD8 in regulating cell survival are possible. FcεRI binding to IgE enhances mast cell survival and CD8 on mast cells could contribute to this effect, perhaps by enhancing mast cell activation and ERK phosphorylation through FcεRI [252].

Modulation of CD8 signaling on NK cells, γδ T cells, and αβ T cells can induce FasL-mediated apoptosis. Evidence suggests activation of monocytes through CD8 and FcR may also regulate monocyte FasL expression. Monocytes and macrophages can upregulate FasL [253-255] upon activation of FcR [256-258] and this may be secondary to release of TNF [259]. Stimulation of human monocytes through FcR and CD8 induces TNF release that may result in expression of FasL by CD8+ rat and human monocytes [31;260]. Thus, ligation of CD8 on monocytes and macrophages may control monocyte apoptosis and tumor cytotoxicity [256;261] via FasL. FcγR on monocytes and other myeloid cells are capable of delivering signals for survival [262;263]. Thus, CD8 on monocytes may, like on T cells, be capable of modulating apoptosis and survival.

6.11.4. A Role for CD8 in Cytotoxic Responses of Monocytes and Macrophages

CD8 on αβ T cells has a well known role in initiating cytotoxic responses and evidence suggests that CD8 on other cells may also promote target cell cytotoxicity. Ligation of CD8αα on NK cells and γδ T cells enhanced release of IFNγ and TNF respectively [185] [184], suggesting a role in promoting cytotoxic responses. Similarly, CD8+ monocytes express cytotoxic mediators like FasL, perforin and granzyme [31;260], and ligation of CD8 promotes expression of TNF [88] and nitric oxide [18], cytotoxic mediators that induce FasL expression [259]. Cytotoxic monocyte and macrophage responses activated by CD8 may be important in transplantation and defense against tumors and intracellular pathogens.

Anti-CD8α mAb treatment of rat macrophages enhanced killing of intracellular *Leishmania* in a nitric oxide dependent manner, but had no effect on phagocytosis of latex particles [31;87]. CD8 protein and CD8+ cells are important in defence against intracellular organisms such as *Haemophilus influenzae*, and tuberculosis in mice [264;265]. Notably a human with a genetic lack of CD8 expression presented with *H. influenzae* lung infection and a history of recurrent lung infection [264]. Given that ligation of CD8 on alveolar macrophages can activate them to kill intracellular organisms [87], it is possible that a lack of CD8 on alveolar macrophages contributed to recurrent lung infections in this individual [264].

There is also a cytotoxic role for CD8+ monocytes in tumor defense. For example, CD8+ rat monocytes infiltrated the site of tumors [30], and exhibited higher anti-tumor cytotoxicity than CD8- monocytes [31]. Immune-complexes in tumors expressing MHC class I may result in co-ligation of CD8 and FcR, and cause tumor cell death by production of TNF, nitric oxide and FasL from monocytes.

Large numbers of CD8+ macrophages and monocytes have been observed in several models of disease in rats including glomerulonephritis [266], arthritis [229], ischaemia [267], severe trauma [268], transplant rejection [32], experimental allergic encephalomyelitis (EAE) [269], autoimmune thyroid disease [260] and myocarditis [31]. CD8+ monocytes proportionally increase after induction of arthritis, myocarditis, or transplant rejection and express markers associated with cytotoxicity (perforin, granzyme B, FasL), NK cells (NKR-P1A, NKR-P2 [NKG2D homolog]), extravasation (CD62L), and macrophage/dendritic cell adhesion (CD11b/c) [31;32;229]. Expression of cytotoxic molecules by CD8+ monocytes correlates well with the ability of CD8 on these cells to cause release of inflammatory/cytotoxic mediators [87;88]. Moreover, the localization of CD8+ monocytes/macrophages correlated with sites of tissue destruction in ischaemia, glomerulonephritis, EAE and myocarditis [31;266;267;269].

Many of these disease models are initiated by immune-complex deposition, and/or FcγR is required for disease pathology. Data in this thesis suggests that co-ligation of FcγR and CD8, as may occur when CD8+ monocytes encounter MHC class I expressing tissues coated with immune complexes, enhances monocyte activation and TNF release. Thus CD8 on monocytes may directly contribute to the severity of many diseases or disease models whose pathology is dependent on immune-complex deposition and FcR activation.

Interestingly, in a model of ischaemia the influx of CD8+ macrophages to the site of necrosis followed peak production of nitric oxide and other cytotoxic mediators, and correlated with the beginning of healing processes [267]. There is no evidence that CD8 on macrophages or CD8+ macrophages release increased amounts of mediators like TGFβ involved in healing or resolution of inflammation [31]. However, while CD8+ macrophages and CD8 on macrophages do not appear to be specialized for phagocytosis of latex particles [18;31], they may specialize in processing cellular debris and apoptotic cells because CD8+ rat monocytes selectively expressed SIRPα, that is involved in recognition and uptake of apoptotic cells [229]. CD8α+ macrophages and DC have a heightened ability to activate CD8+ T cells to respond to antigens from viruses, intracellular bacteria [270] or apoptotic cells and presented on MHC class I [271;272].

Interestingly, ligation of MHC class I or MHC class II induces signaling through Syk kinase/PI3K/PKC in the antigen-presenting cell[273-276], and CD8 associates in the cell membrane with MHC class I [33]. It is plausible, that CD8 signaling through LAT could amplify activation of antigen-presenting cells through MHC class I. Quantitative or qualitative enhancement of signaling via CD8 in concert with MHC class I through Syk/PI3K/PKC may modulate the phenotype of CD8+ antigen presenting cells and modify their ability to activate the next T cells they encounter. CD8+ macrophages may regulate how intracellular antigens are presented to generate CTL, regulatory T cells, or potentially eliminate T cells through a veto effect.

6.12. Mistaken Identities: CD8+ Monocytes, Macrophages, and T cells

CD8+ monocytes and CTL are often present together at sites of injury and disease [30;266] [229] [267] [268] [32;269] [260] [31]. Moreover they may have similar effects, for example, mediating a veto effect or cytotoxicity and tissue destruction. Whether on dendritic cells, monocytes, B cell lymphoma, veto cells of uncertain lineage or NK cells, CD8 is often co-expressed with several proteins frequently used as phenotypic identifiers of T cells such as CD3δ and CD3ε, granzyme B, perforin, and even preTCRα and TCRβ (see introduction "Common Traits") [218;219] [215] [220] [221-226]. As a consequence it is possible, even likely, that CD8+ monocytes are mistakenly identified as CD8+ CTL at times, particularly because it is not widely appreciated that monocytes and macrophages express CD8. In fact, since the expression of CD8 by rat monocytes and macrophages has become more widely known such mistaken identification of CD8+ monocytes and CTL has been documented several times [30;266] [229] [267] [268] [32;269] [260] [31].

For example, CD8+ monocytes and macrophages, not CD8+ T cells were primarily responsible for tumor killing and regression in one model [30]. In this study tumor infiltrating macrophages were CD8+ and killed tumor cells via nitric oxide [30]. Since CD8 on macrophages can induce nitric oxide production [87], CD8 could be directly involved in this tumor cytotoxicity. T cell infiltrates into the tumors were important for IFNγ production, but not direct cytotoxicity [30]. Addition of IFNγ alone stimulated macrophage cytotoxicity [30].

Akin to the aforementioned tumor model it is possible that in many diseases where CD8+ T cells and monocytes infiltrate the site of disease, CD8+ T cells may activate macrophages through IFNγ, and the macrophages may then effect tissue damage. This may be the case in some models where depletion of CD8+ cells [277] or macrophages [278] mitigates tissue pathology, such as glomerulonephritis [278]. It will important to re-evaluate the identity and roles of CD8+ cells in many rat models and human diseases where this has not been thoroughly performed with markers for TCRα.

6.13. FUTURE DIRECTIONS

While our understanding of the expression and function of CD8 on monocytes and macrophages has advanced, many significant gaps remain. In the following section I will outline some of the notable gaps and propose experiments to respond to these gaps. The proposed experiments are an enormous undertaking that cannot be performed by one laboratory alone.

While mouse NK cells and monocytes appear to be CD8α(-), the same cells in human and rat are CD8α+. Thus, a comparative analysis of CD8 expression by NK cells and monocytes from mice and humans performed concurrently would help solidify evidence of apparent species differences in CD8 expression.

CD8 expression is controlled largely by regulation of DNA accessibility by heterochromatin formation. Examining Ikaros expression, DNA methylation, or DNA hypersensitivity sites may indicate the mechanism controlling the low level of constitutive CD8 expression in monocytes versus the high level of CD8 expression on T cells. Finally, CD8 is post-transcriptionally regulated during T cell development and on peripheral CD4+CD8α+ T cells

[279-281]. MicroRNAs are 21-26 nucleotide RNA molecules that post-transcriptionally direct gene expression via DNA heterochromatin formation, mRNA cleavage or suppression of mRNA translation in a sequence specific manner. Overexpression of mouse microRNA 181 decreases the number of CD8+ cytotoxic T cells [282], a phenotype also observed in CD8 null mice [283]. Bioinformatic analysis of the CD8α gene (Ensembl identifier: ENSG00000153563) region for targets of known microRNAs with miRBase reveals that six other human miRNAs (has-107, -103, 377, -615, -297b, and 346) may regulate CD8α expression.

A high level of CD8 protein has been described on a subpopulation of monocytes/macrophages in blood or at disease sites when inflammation is ongoing [30;266] [229] [267] [268] [32;269] [260] [31]. Is CD8 in these situations synthesized by monocytes/macrophages and capable of initiating intracellular signaling and cell activation, or is it not functional? For example, soluble CD8 secreted by activated T cells may be adsorbed to the monocyte/macrophage surface, as it is to the dendritic cell surface [284] and be functionally inert. Isolation and short-term culture of monocytes from some of these disease models could be used to scrutinize CD8α mRNA expression and CD8α re-synthesis after trypsin cleavage. Evidence suggests GM-CSF and FLT-3 may be directly or indirectly involved in upregulating CD8α expression on myeloid cells *in vivo*. Testing whether these soluble factors can upregulate CD8 on monocytes and macrophages *in vitro* may provide a foundation for the possibility that CD8 is expressed at high levels on monocytes and macrophages in some inflammatory settings, and direct future investigation of CD8 on monocytes in particular diseases.

While these studies suggest for the first time that CD8 may also signal through common γ chain-linked receptors like FcR, a thorough understanding of the mechanisms of CD8 function on monocytes and NK cells is distant. First a re-examination of CD8 and TCR signaling proteins in monocytes and macrophages with contemporary sensitive techniques is warranted given some evidence that CD3 chains and lck can be found in macrophages and dendritic cells. Second, the possibility that CD8α on monocytes binds hck, as evidence suggests for CD4 should be examined [53]. Third, it is necessary to re-visit evidence that CD8 directly binds LAT to better understand how CD8 may signal in monocytes and T cells. To exclude indirect binding of LAT to CD8 via lck, one could transfect CD8(-)/lck(-) cells with LAT and CD8 wild type, CD8 lacking the cytoplasmic domain, and CD8 mutated in the CxC motif implicated in lck and LAT binding. This could be supported by analysis of binding of recombinant LAT to the CD8α cytoplasmic domain in a cell-free environment. These studies would allow an understanding of the possible options for CD8α signaling in monocytes. Subsequent studies could analyze CD8α interactions in human monocytes with signaling proteins proposed by the above studies, and examine phosphorylation events following CD8α ligation.

It is not clear which FcγR receptor(s) CD8α may interact with. The reliance on anti-CD8α mAb to activate CD8α makes some approaches problematic. Studies could employ cross-linking CD8α with a variety of common γ chain-linked receptors, including each FcR using specific mAb, and potentially using siRNA knockdown to consolidate the dependence of responses on each FcR and CD8α. It will be important to examine FcR ligation and CD8α ligation with MHC class I or MHC class I mutated to eliminate binding to CD8. This approach will begin to delineate the contribution of CD8 to the competing balance of activating and inhibitory monocyte receptors that bind MHC class I. One might predict this balance is modulated in different disease settings.

A powerful alternative to carefully dissect FcR-CD8 signaling may be to reconstitute FcR signaling and responses in transfected cells normally negative for these proteins, as done by others [285;286]. The effect of CD8 transfection and ligation with MHC class I on FcR responses could then be scrutinized. These kinds of studies could be extended to examine the effect of CD8 ligation on responses of other receptors that use the common γ chain (PIR-A or ILT1) or DAP12 (SIRPβ). This combination of studies may allow the dissection of the mechanisms and spectrum of ways in which CD8 may enhance responses of monocytes or T cells.

As PIR-A and ILT1 likely bind MHC class I or related proteins, it will be interesting to learn if their binding sites on MHC class I overlap with that of CD8α. Moreover, according to models proposed in the section "CD8: Re-examining the Co-Receptor Concept" it is possible that CD8 could enhance binding of PIR-A or ILT1 to MHC class I, particularly if they have slow on-rates of binding like TCR. A photoreactive protein-protein cross-linker has been used to study the ability of CD8 to promote binding of TCR to MHC class I. By adapting the attachment site of the cross-linker on MHC class I, similar experiments may be used to study the effect of CD8 on binding of PIR-A, ILT-1, Ly49 or other receptors to MHC class I.

Finally, the functions of CD8(+) monocytes and macrophages in inflammatory sites in several disease models with acute immune-complex and FcγR dependent injury must be established. CD8(+) monocytes/macrophages and CD8(+) T cells may be retrieved and separated from rat tumor or glomerulonephritis models, or potentially from patients with similar diseases. Not only would this allow independent analysis of CD8(+) T cell and monocyte functions, but also the role of CD8 on monocytes in these diseases through *in vitro* analysis of their responses to FcR ligation and CD8 ligation with MHC class I and MHC class I mutated in the CD8 binding site. Monocyte and macrophage phenotype and responsiveness is heterogeneous and plastic depending on their environment. The proposed experiments would assess the influence of CD8 on monocyte signaling and functions and link these findings to monocyte responses in disease. Further studies could refine the range of monocyte responses influenced by CD8, including cytokine release, radical production, maturation to antigen-presenting function, and mechanisms of cytotoxicity, healing, monocyte apoptosis or survival.

6.14. CONCLUSIONS: TOWARDS A CD8 WITHOUT THE TCR

Careful examination of the literature suggested the expression of CD8 differed among rat, mouse and human species, and hinted that despite the lack of CD8 expression on mouse macrophages and monocytes, CD8 might be expressed by human monocytes and macrophages. I have demonstrated the expression of CD8α on human monocytes, thus extending evidence that the expression pattern and other characteristics of CD8 vary significantly among species (Table 1-1 and 1-2).

By building on evidence of how CD8 participates in T cell activation I have expanded current models of how CD8 may function in CD8+ cells that do not express TCR (see Figure 1-9, Table 1-3). These models predict CD8 enhances signaling of many receptors on monocytes, NK cells or other cells, although potentially not as robustly as the enhancement of TCR responses by CD8 on CTL. Evidence predicted the best candidate for CD8 co-activated responses would be FcR, potentially signaling via LAT. I have tested these predictions and provide evidence that CD8 expressed by monocytes enhances phosphorylation of LAT, and cell responses through FcR. This is the first evidence attributing a co-activating role to CD8 with a receptor other than TCR.

Like CD8α, many receptors may activate lck (CD28 [287], NKRP-1 inhibitory and activating receptors [288], CD94 [289], and FcR [290]) or LAT (2B4 [188], CD2 [189]) and bind other cells. CD8 may activate intracellular signaling pathways distinct from those of some cell surface receptors that co-activate TCR responses, like integrins [124]. CD8 may or may not have a unique function in activation of CTL and other cells, whether due to its binding rates or binding site for MHC class I, association with other receptors, timing of recruitment to the site of cell activation, or association with signaling molecules. Other differences in how receptors activate T cell responses may include quantity, precise timing of recruitment, and duration or oscillatory rhythm of their effects on intracellular signaling [143;291;292].

Several immune receptors, like FcR, may bind ligands found on pathogens like bacteria, on cells, or in solution. Detecting a conserved motif in a ligand (MHC class I) expressed on most cells, CD8 may help to elicit the optimal response to cell-associated stimuli, (whether self, allogeneic, infected or tumorigenic cells) in comparison to responses specialized to eliminate extracellular pathogens, inert particles, or debris that is MHC class I(-ve) and would not engage CD8 (Figure 6-10). CD8α may co-operate with diverse immune activating receptors that use Syk/ZAP-70 and CD3ζ or common γ chain. A prediction of this model is that CD8 would contribute to heightened NK cell and monocyte responses in FcR-dependent antibody-dependent cell-mediated cytotoxicity (ADCC, Figure 6-10). This may be relevant to autoimmune diseases like lupus and glomerulonephritis, and tumor and anti-viral defense [175]. Therapeutic targeting of CD8 could be appropriate in a range of diseases where the aim is to quell FcR-dependent inflammation without rendering the patient profoundly immunodeficient. Additionally, one might hypothesize that connection of a CD8-stimulating agent to monoclonal antibodies that bind tumors in cancer therapies like rituximab (anti-CD20) and trastuzumab (anti-Her2/Neu) may increase the efficacy of tumor killing by NK cells and monocytes.

6.15. Tables

Before Thesis

	CD8α		CD8β	
	NK cell	Monocyte	NK cell	Monocyte
Human	+ [7-10]	? (P) [31;293]	- [8]	?
Mouse	- [7]	- [5]	-	-
Rat	+ [11;12]	? (P) [31;294]	- [12]	? (P) [31]

After Thesis

	CD8α		CD8β	
	NK cell	Monocyte	NK cell	Monocyte
Human	+ [7-10]	+	- [8]	-
Mouse	- [7]	- [5]	-	-
Rat	+ [11;12]	? (P) [31;294]	- [12]	? (P) [31]

Table 6-1. Contribution of this thesis to the understanding of CD8 expression on monocytes, and species differences in the cell types that express CD8. The expression of CD8α and the lack of expression of CD8β by human monocytes is highlighted. Expression of CD8 is denoted by a + symbol where evidence of synthesis of CD8 by the cell type in the given species is available. Number of + symbols denotes relative level of protein on cell surface. (P) denotes situations where protein has been shown on the cell surface, but there is no evidence that the protein is synthesized by the cell.

Interaction	Temp (°C)	Kd (µM)	Kon (M-1•s-1)	Koff (s-1)
TCR-MHC	25	1-10	[redacted]	0.01-0.2 [151]
CD8-MHC class I	25	50-200	1.4 x 105	>20 [22;151;295]
KIR-MHC class I	25	10	2 x 105	2 [146]
ILT2/4-MHC class I	25	2-45		
Ly49A-MHC class I	25	10	[redacted]	0.03 [296]
FcRII/FcRIII	25	0.65-2.5	3.2-5.4 x105	0.27-1.1 [148]
CD2-CD58	37	10	4 x 105	4 [297]
CD2-CD48 (rodent)	37	60	>105	6 [298]
2B4-CD48	37	10	3 x 105	3[299]
CD28-CD86	37	20	106	>20 [300]
CTLA-4-CD80	37	0.2	2 x 106	0.4 [301]
OX2-OX2R	37	2	4 x 105	0.8 [302]
Selectin-ligand	37	0.3-100	105-106	1.4-10 [303]
LFA-1-ICAM-1	25	0.13	2 x 105	0.03 [304]

Table 6-2. The fast on-rate (K_{on}) of CD8 may promote binding of receptors with slow on-rates like TCR. Few MHC class I binding receptors with known binding rates, have slow on-rates like TCR, with the exception of Ly49. CD8αα and CD8αβ bind with the same characteristics, but there is some difference between alleles. TCR:peptide-MHC Kd values at 37°C are typically 1.5–2.5 fold higher than at 25°C. Parts of table are taken from van der Merwe PA and SJ Davis, *Annu Rev. Immunol.* (2003) 21: 659

Cytotoxicity	References
Perforin	[31;217]
Granzyme	[31;217]
TCR Signaling	
CD3εδ	[220;221;228]
Pre-TCRα	[221]
Lck	[215;305;306]
LAT	[172;307]
ZAP-70	[218;219]
TCR Re-arrangement	
RAG1/2	[221]
Ig-H D-J Re-arrangement	[221]

Table 6-3. T cell or lymphocyte "specific" proteins/mRNA detected in CD8+ cells that are not lymphocytes. CD8 expression may occur as part of a broader transcriptional program that controls expression of proteins involved in TCR-CD8 signaling or cytotoxicity through changes in DNA methylation and euchromatin/heterochromatin. Non-lymphocytic cells studied in references in the table include dendritic cells, monocytes, and megakaryocytes. ZAP-70, lck, and CD3δε chains are considered T cell-specific, but have been identified in some myeloid cells and B cell lymphomas.

6.16. Figure Legends and Figures

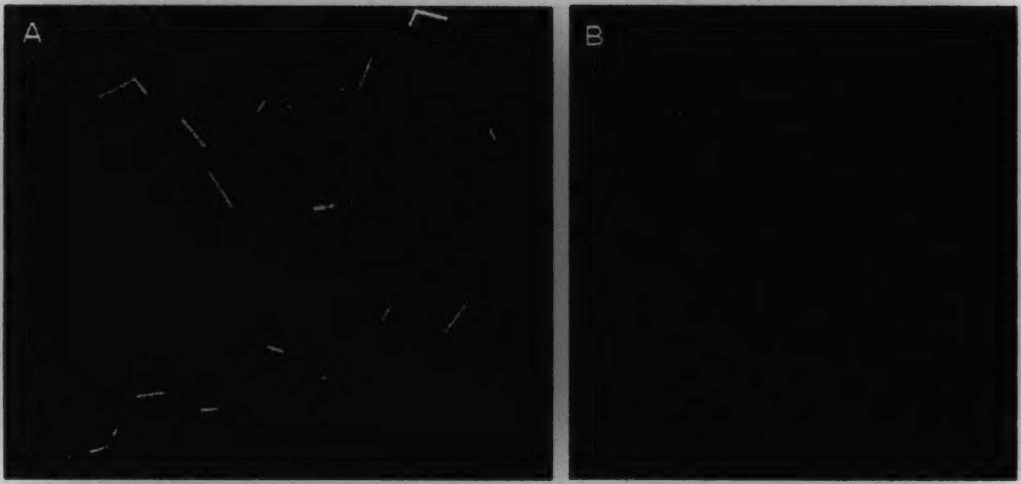


Figure 6-1. Cysteine residues and disulphide bonds of CD8α. In crystal structures of CD8αα the canonical Ig superfamily disulphide bond between β-sheet strands B (C26) and F (C102) is observed. B-F disulphide is shown in (B) by red solid line (human CD8αα IAKJ). Using different methods of analysis others have observed disulphide bonds in CD8α between B and C (C36 in panel A) strands. The C strand cysteine is highlighted in yellow in B, and a hypothetical disulphide bond is shown by a dashed red line. (A) is taken from Kern PS et al. *Immunity* (1998) 9: 519. (B) was generated with Cn3D program.

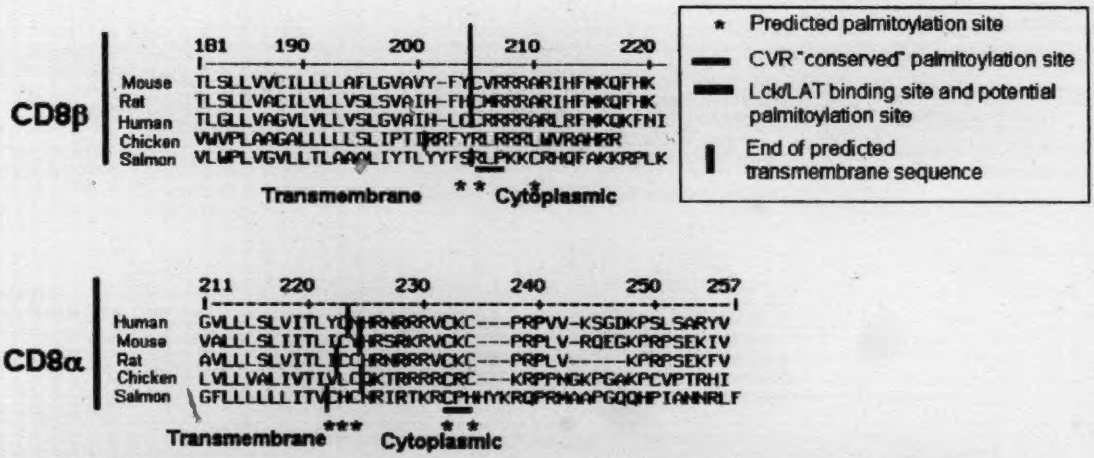

CD8β
181
190
200
210
220
Mouse TLSLLVVCILLLLLAFLGVAVY-FYCVRRRARIHFMKQFHK
Rat TLSLLVACILVLLVSLSVAIH-FHCMRRRARIHFMKQFHK
Human TLGLLVAGVLVLLVSLGVAIH-LCCRRRRARLRFMKQKFNI
Chicken VWVPLAAGALLLLLSLIPTIRRRFYRLRRRLWVRAHRR
Salmon VLWPLVGVLLTLAAALIYTLYYFSRLPKKCRHQFAKKRPLK
Transmembrane
* *
*
Cytoplasmic
★ Predicted palmitoylation site
CVR "conserved" palmitoylation site
Lck/LAT binding site and potential palmitoylation site
End of predicted transmembrane sequence
CD8α
211
220
230
240
250
257
Human GVLLLSLVITLYCNHRNRRRVCKC---PRPVV-KSGDKPSLSARYV
Mouse VALLLSLIITLICYHRSRKRVCKC---PRPLV-RQEGKPRPSEKIV
Rat AVLLLSLVITLICCHRNRRRVCKC---PRPLV------KPRPSEKFV
Chicken LVLLVALIVTIVLCCKTRRRRCRC---KRPPNGKPGAKPCVPTRHI
Salmon GFLLLLLLITVCHCHRIRTKRCPHHYKRQPRHAAPGQQHPIANNRLF
Transmembrane
***
* *
Cytoplasmic

Figure 6-2. Palmitoylation of some sites in CD8α would exclude CD8 binding to Lck or LAT. All potential palmitoylation sites in CD8α and CD8β are shown (cysteine residues within 12 amino acids of the membrane, or just within the membrane may be palmitoylated {994}). Some palmitoylation sites in CD8α overlap the lck/LAT binding motif. CD8β sequences used: mouse (NP_033988), rat (P05541), human (NP_742097), chicken (NP_990578), salmon (AAW23970). CD8α sequences used: human (NP_001759), mouse (P01731), rat (P07725), chicken (NP_990566), salmon (AAW23969). Transmembrane predicted with TMpred at embnet.org (Hofman and Stoffel Biol. Chem [1993] 374, 166).

Lipid raft Partitioning	+++	++	++	+++
MHC class I binding	+	+	+	+
Lck/LAT signaling	++	+	—	—
Association with TCR	—	+	+	—

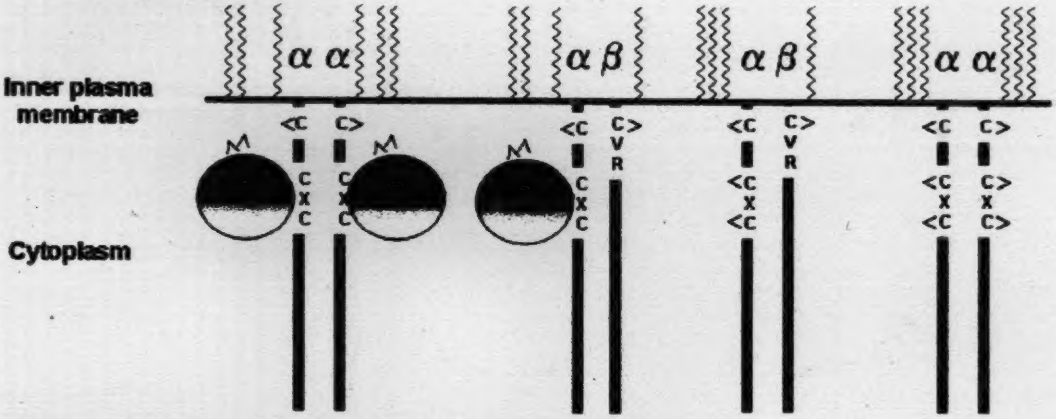

α α
α β
α β
α α
Inner plasma membrane
Cytoplasm
<C C>
C X C
<C C> V R
<C X <C
<C C> <C X <C C> X C>

Figure 6-3. Hypothetical CD8 dimers that may allow separation of CD8 functions. Only cytoplasmic domains of CD8 are shown. Amino acids are represented with single letter code. Hypothetical abilities of each CD8 dimer shown are semi-quantitatively represented by + symbols. CD8β is believed to allow CD8 to associate with TCR.

⌇	**Palmitoyl group**
<	**Palmitoylated cysteine**
α, β	**CD8 chains**

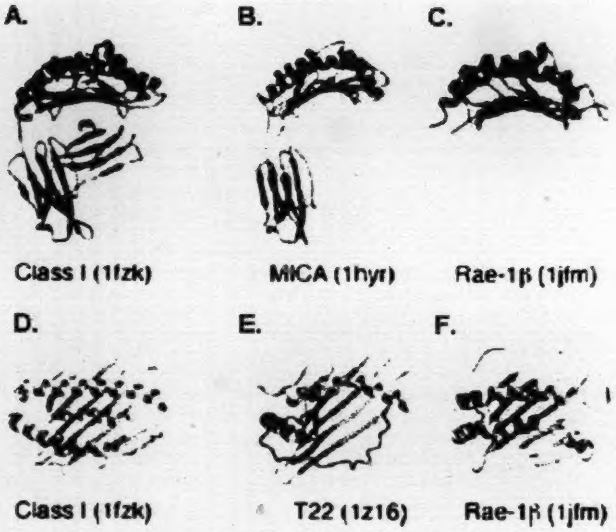
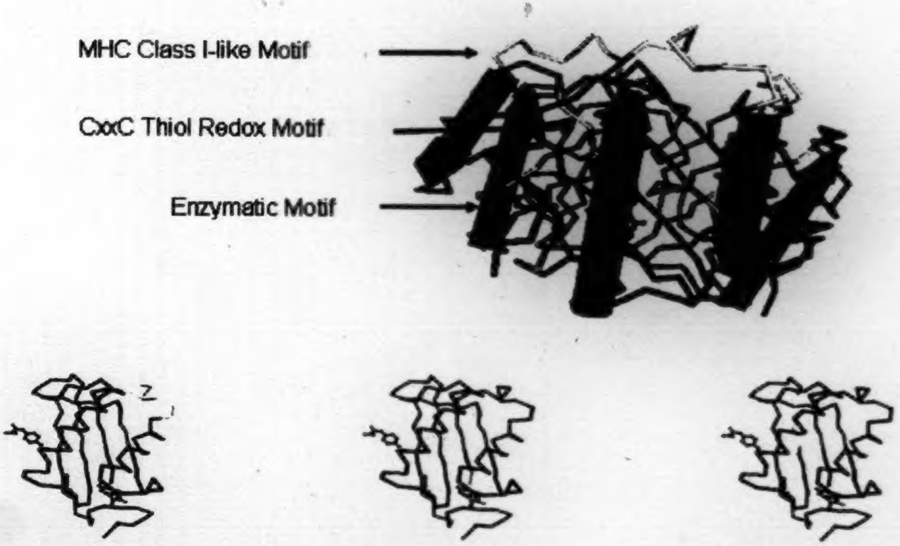


Figure 6-4. Some non-classical MHC class I molecules lack significant parts of the classical MHC class I structure. (A-C) MHC class I proteins are shown in blue, attached β2m is shown in brown. (A) Classical MHC class I (B) MIC-A does not bind β2m. (C) Rae-1β does not contain the α3 domain or bind β2m. (D-F) show the groove formed by α-helices of MHC class I proteins, from the perspective of the TCR. MHC class I molecules are in grey. (D) Classical MHC class I with peptide bound in groove. Non-classical MHC class I molecules (E) mouse T22, and (F) Rae-1β lack significant portions of the α helices and α1 α2 domains found in classical MHC class I. Figure adapted from Rudolph MG et al. *Ann. Rev. Immunol.* (2006) 24: 419.



MHC Class I-like Motif (11-18) Enzymatic Motif (1-2, 95) CxxC Thiol Redox Motif (57-60)

Figure 6-5. Spatial separation of functional motifs of MIF. Three motifs in MIF (highlighted in yellow) have been attributed distinct functions. Surface-exposed loops contain a MHC class I-like motif PRASVPEG, and have been suggested to bind receptors (Leng, L. *JEM* (2003)197: 1467. MIF also contains a thiol redox motif and an enzymatic tautomerase motif. Top, MIF in trimeric, secreted form. Bottom, three panels show monomeric MIF. Key residues of given motif are noted in brackets. Structures (1MFI) show the small molecule inhibitor E-(2)-fluoro-2-hydroxycinnamate bound to the enzymatic site.

Co-Receptor

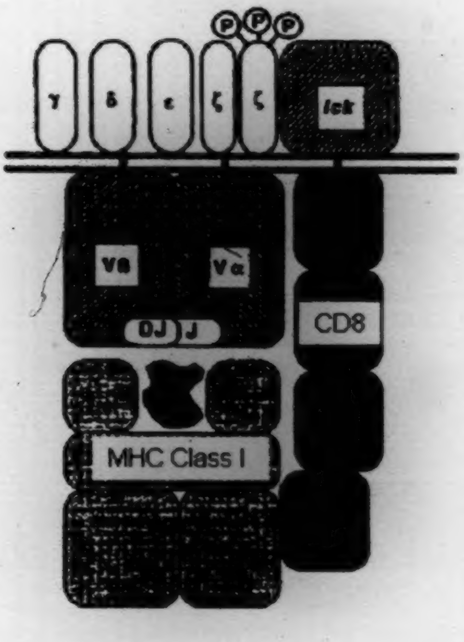

P
P
P
γ
δ
ε
ζ
ζ
lck
Vβ
Vα
DJ
J
CD8
MHC Class I

Figure 6-6. Classical co-receptor model of CD8 showing CD8 binding the same MHC class I as TCR. Adapted from Janeway CA Jr. *Annu. Rev. Immunol.* (1992) 10: 645

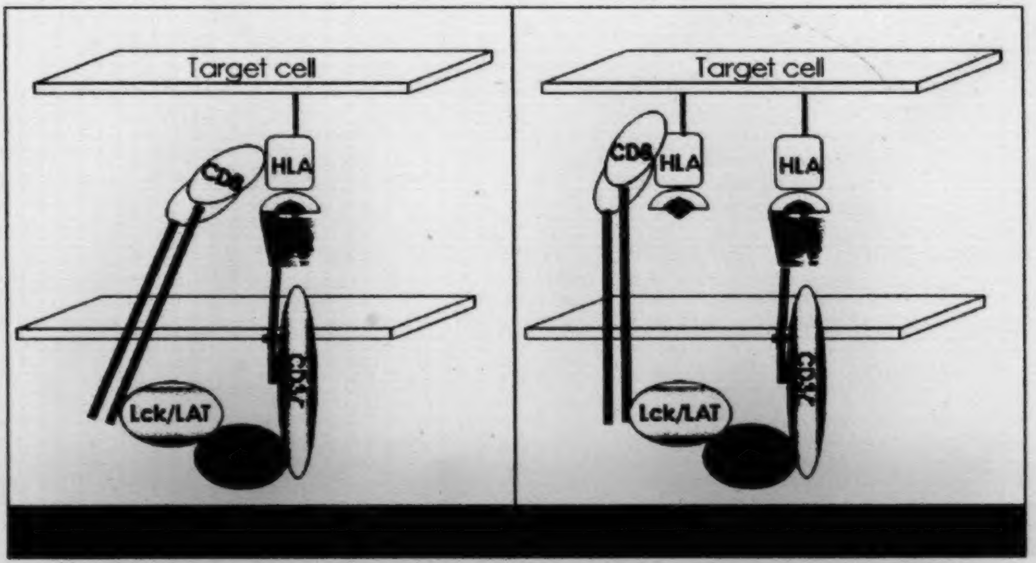

Target cell
CD8
HLA
CD3γ
Lck/LAT
Target cell
CD8
HLA
HLA
CD3γ
Lck/LAT

Figure 6-7. Two models of CD8 enhancement of TCR responses. Co-receptor model of CD8 (left): Optimal enhancement of TCR responses by CD8 is proposed to require CD8 and TCR simultaneously binding the same MHC class I molecule. Co-activation model of CD8 (right): CD8 may promote TCR binding to MHC class I, and co-operate in TCR signaling without binding the same MHC class I as TCR.

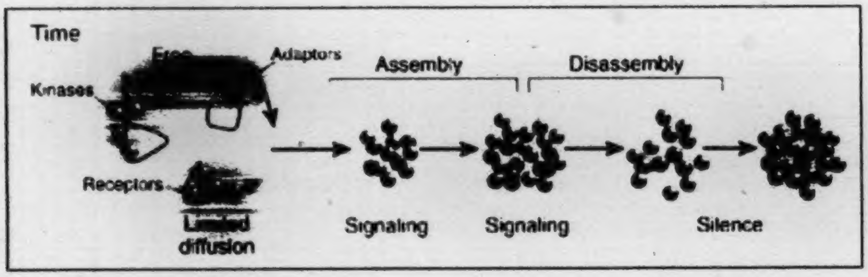

Time
Free
Adaptors
Kinases
Receptors
Limited
diffusion
Assembly
Disassembly
Signaling
Signaling
Silence

Figure 6-8. Speeds and pauses: theory of cell activation by limiting diffusion. Free and rapid diffusion is slowed and limited by transient low affinity interactions of receptors, kinases and adaptor molecules. If a threshold is reached, activated microclusters of signaling complexes can aggregate further and induce cell activation. The model allows that CD8 may be recruited to participate in cell activation via lck, LAT, MHC class I or a direct association with TCR. Taken from Trautmann A., *Nat. Immunol.* (2005) 6: 1213

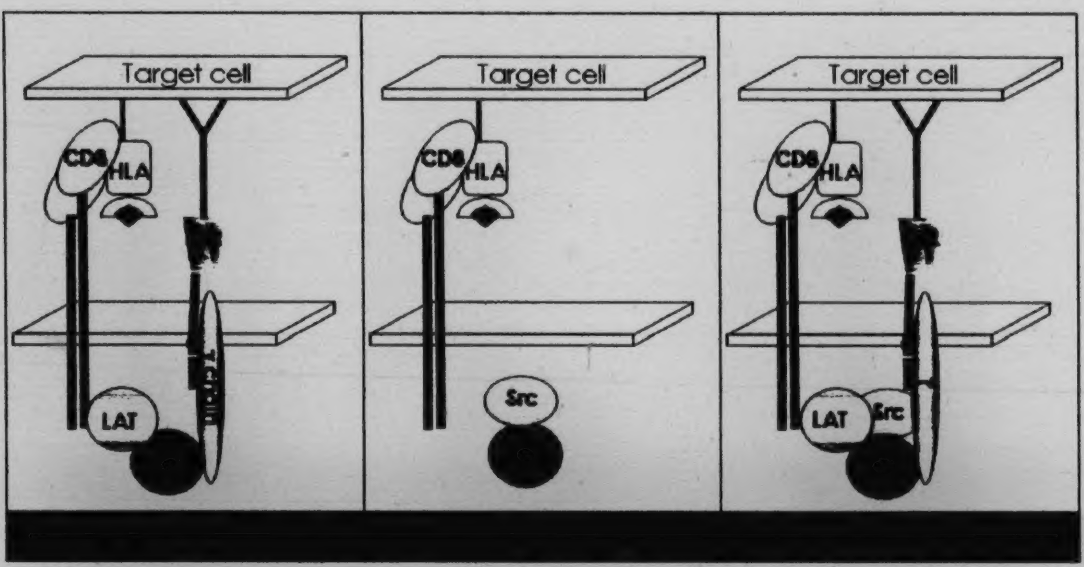

Target cell
CD8
HLA
LAT
Target cell
CD8
HLA
Src
Target cell
CD8
HLA
LAT
Src

Figure 6-9. Contribution of this thesis to models of CD8 signaling mechanisms and co-activation on monocytes and macrophages. Previous to this thesis ("Evidence Before") CD8 activation of rat macrophages was known to depend on an unidentified src kinase (that may not bind CD8), and Syk kinase. Studies in this thesis suggest CD8 on human monocytes enhances phosphorylation of LAT, and activates TNF release in an FcR-dependent manner. Whether CD8 directly binds LAT or other molecules is hypothetical, but the "Evidence After" thesis model is based on previously described interactions of CD8 and FcR signaling.

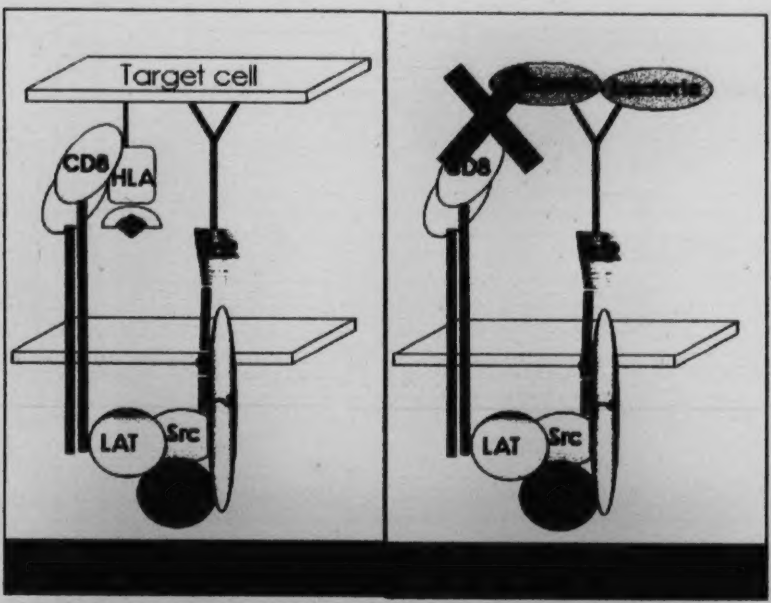


Figure 6-10. CD8 may enhance monocyte responses to cellular stimuli. Co-ligation of CD8 with FcR may enhance monocyte antibody-dependent cell-mediated cytotoxicity in response to MHC class I expressing cells coated with immunoglobulin (e.g. viral infection, autoimmunity, tumor defense). In contrast monocyte responses initiated by FcR against acellular stimuli such as extracellular bacteria or soluble immune complexes may not be enhanced by CD8.

6.17. Literature Cited

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7. APPENDIX 1. CONCERNING THE SPECIALIZATION OF CD8αα AND CD8αβ

Debate surrounds the roles of CD8αα versus CD8αβ as a TCR co-activator (e.g. [1;2]). Some theories have even proposed that CD8αα inhibits CTL activation, while only CD8αβ can enhance CTL responses [2]. Human monocytes, as well as NK cells and dendritic cells express only CD8αα (Table 1-1, Part I), and thus, if CD8αα (only) inhibits cell activation, it directly contradicts evidence and models presented in this thesis that CD8αα enhances monocyte FcγR activation. Because of this potential contradiction I will discuss existing models of CD8αα function on T cells, and provide evidence that CD8αα promotes T cell activation. This will thus support the model that CD8αα enhances monocyte FcR responses in ways similar to CD8αα enhancement of TCR responses. Furthermore, I suggest that the current study adds to work suggesting that the ability to elucidate the heterogeneity of physical characteristics and function of CD8 is artificially limited if it is restricted to distinguishing CD8αα and CD8αβ.

One might expect that two dimeric forms of CD8, CD8αβ and CD8αα, exist to fulfill two different functions, whether subtly or drastically different. Quantitatively CD8 can enable T cells to respond to up to 10^6 less MHC class I-peptide complexes [3;4]. CD8αβ allows T cells to respond to 10-100 fold less MHC class I-peptide than CD8αα, suggesting CD8αβ has a heightened ability to activate T cells in comparison to CD8αα (10^6 for CD8, 10-100-fold for CD8αβ) [5]. Lack of CD8β reduces the number of positively selected mature T cells to 25%

of normal [6-9], suggesting a role for CD8β, at least in positive selection, that cannot be wholly substituted by CD8α.

Through two identified means CD8β promotes CTL activation [5;9-14] and CD8αβ appears in many situations to be a better co-activator of CTL than CD8αα. Sialylation of the stalk region of CD8β regulates MHC class I binding and T cell activation of thymocytes [15]. CD8αβ seems to found in close proximity with TCR on the cell surface in greater proportions than CD8αα, and this may allow CD8αβ to more efficiently promote binding of TCR to MHC class I [13;16;17]. These enhanced abilities of CD8αβ in T cell activation along with the high affinity of CD8αα but not CD8αβ binding to the MHC class I molecule TL [18] has fostered much debate that CD8αα has functions distinct from CD8αβ. Without a TL homologue known in human [19] it is possible CD8αα interaction with TL is a species-restricted anomaly.

If CD8αβ is proposed to be a better TCR co-activator than CD8αα, or even the only TCR co-activator, the role that CD8αα is specialized to fulfill is unclear. Perhaps CD8αα and CD8αβ have partly segregated roles in promoting cell activation. For example, CD8αβ may specialize in promoting binding of TCR (or other receptors) to its ligand, and CD8αα may specialize in other components of cell activation. Recently three theories have been put forward to attempt to construct a signatory function for CD8αα. All three theories, however, have their weaknesses.

7.1. Theory 1. CD8αβ Co-Receptor/CD8αα Anti-Co-Receptor: Opposites are Often the Same

One of these theories suggests CD8αα is an "anti-co-receptor" [2], that downregulates T cell responses by sequestering lck from lipid raft based signaling of the TCR [2;18;20-22]. The theory that CD8αα is an anti-co-receptor is based on the observation that ligation of CD8αα on intestinal epithelial lymphocytes (IEL) slightly decreases their proliferation and cytotoxicity, but increases their IFNγ release [23]. However, outside this one study there is little or no evidence that CD8αα inhibits T cell responses. Rather, as noted in the introductory section of the appendix, CD8αα is sometimes a less efficient co-receptor than CD8αβ, but not always. In other studies, CD8αα and CD8αβ activate equivalent responses from CTL, even across a broad range of TCR affinities [24]. In other models CD8αα transfectants have responses similar to those of CD8αβ transfectants [25]; or a small increase in IL-2 production [26] found in CD8αβ transfectants correlates with increased expression of CD8α when CD8β is co-transfected [25;26]. Similarly, while CD8αβ may robustly induce lck phosphorylation compared to CD8αα, CD8αα induces some lck phosphorylation, and induces nearly equivalent phosphorylation of CD3ζ [11]. At the very least, many studies have shown CD8αα enhances or reconstitutes T cell activation [25;27-29] and binding of TCR to MHC class I tetramers [12;13;30;31]: two functions intimately associated with CD8 co-activation of CTL.

After a base threshold for activation through TCR is reached, T cell responses increase exponentially [32]. As such, depending on the titratable point on the exponential curve that studies are performed with CTL clones or a single TCR, small initial differences can become all or nothing differences in CTL cytotoxicity, proliferation or cytokine release. When large differences between CD8αα and CD8αβ have been found, T cell hybridomas with a single TCR have often been used without dose-responses (as in studies of CD8β palmitoylation [11;33] and association with CD3δ-TCR [13]. Indeed, if CD8αβ is solely responsible for CD8 association with TCR and enhancement of TCR binding to MHC class I as proposed [11-13;33], it is puzzling that CD8αα increased binding of MHC class I to TCR to the same extent as CD8αβ in previous studies using the same technique [30;31]. Rather than CD8αα being an "anti-co-receptor" it seems more considered to suggest that CD8αβ heterodimers are specialized to greatly aid positive selection and perhaps, but not always, other stages of CTL activation. CD8αα appears capable of co-activating responses of CTL. This suggests the model that CD8αα on monocytes can co-activate responses initiated by FcR is valid.

7.2. Theory 2. CD8αα and T Cell Memory Failures

CD8αα is argued to be functionally distinct from CD8αβ [1;2] because one study [34] suggested CD8αα is necessary for generation of T cell memory. It is clear that CD8αα is expressed on a large proportion of memory T cells [18;34-36]. However, the single study that claims CD8αα is required for generation of T cell memory [34] has been heavily challenged by several other groups [18;35;36]. The original study [34] used mice with a deleted CD8α enhancer region, E8(I)-/-, which were thought to be deficient in expression of CD8αα on activated T cells that may become memory T cells. Others, in contrast, found that CD8αα expression on activated peripheral T cells is normal in these mice [35], suggesting that it is an inappropriate model to examine the contribution of CD8αα to the generation of T cell memory. Moreover two groups using the same E8(I)-/- mice, and even the same virus and route of infection as the original study found normal peripheral T cell memory generation

[35;36]. Thus, even if E8(I)-/- mice do have defective memory T cell generation this is likely to be related to regulation of genes other than CD8. Finally, lack of TL, the high affinity CD8αα ligand proposed to be required for memory T cell responses [34] had no effect on memory T cell generation; a single classical MHC class I could induce equivalent T cell memory [37]. Thus at this time CD8αα expression only correlates with IL-7R+ Bcl-2 upregulated memory T cell precursors [34]. A causative link between CD8αα and memory T cell generation is lacking.

7.3. Theory 3. CD8αα versus CD8αβ: not so strict distinctions

The third theory has suggested that CD8αα is not a functional homologue of CD8αβ [1]. However, the argument amplifies differences between CD8αα and CD8αβ, while not mentioning other differences in CD8 that do not clearly segregate with CD8αα or CD8αβ. For example, CD8αβ changes its affinity/avidity for MHC class I significantly on immature mouse thymocytes [15;38], and after CTL activation, increasing on CTL clones, while decreasing on naïve T cells through changes in glycosylation and possibly conformation [39;40]. Perhaps this is considered optimization of CD8αβ function to the situation. In contrast, rather than the higher binding affinity of CD8αα for TL being an optimization of CD8 and CTL responses to certain TL-expressing cell types like activated T cells, epithelial cells and dendritic cells [18] CD8αα is functionally distinct from CD8αβ [1]. In a similarly exclusive logic, because CD8αα is expressed on cells without an MHC class I restricted αβ TCR it has been suggested that CD8αα must have a different function than CD8αβ [1]. As outlined above, CD8αα is also expressed by many CD8αβ+ CTL [14], and CD8αβ is expressed on cells without an αβ TCR (mouse and rat γδ T cells, rat macrophages, mast cells; Table 1-1 Part II).

The distinctions drawn between CD8αα and CD8αβ are not very robust. In fact, given the potential complexity of CD8 regulation by palmitoylation (Figure 6-3) and glycosyl heterogeneity (described in chapter 2, and by others using 2-D electrophoresis [41;42] mass spectrometry and chromatography [43]), that alter CD8 binding affinity/avidity for MHC class I in several circumstances [15;38;39] it may be apt to suggest that multiple forms of CD8 provide subtle regulation of T cell and monocyte activation via rates of association with lck, LAT, lipid rafts and MHC class I.

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8. APPENDIX 2. CD8 ASSOCIATES WITH TCR AND POTENTIALLY OTHER RECEPTORS

8.1. CD8 May Associate with CD3δ and TCR

Some evidence suggests a portion of total cellular CD8, mostly CD8αβ associates with TCR before activation on some subsets of CTL. This precocious association of CD8 with TCR, as would be expected, promotes TCR binding to antigen-specific MHC class I, early formation of intracellular signaling complexes and thereby CTL responses to lower doses of antigen-specific MHC class I [1-4]. However, it is difficult to conclude that CD8-CD3-TCR association is not due to co-segregation into lipid rafts, or driven by binding of intracellular signaling proteins.

Constitutive association of CD8 with the TCR-CD3 complex was detected by co-immunoprecipitation and fluorescence resonance energy transfer (FRET) in mouse T cell clones, thymocytes, and memory T cells, but not naïve peripheral T cells [1-4]. Other studies attempted to delineate the regions of CD8 mediating association with the TCR-CD3 complex. The cytoplasmic region of CD8β promoted the co-immunoprecipitation of CD8 with CD3δ and CD3ε [3;5]. CD3δ in turn promotes co-immunoprecipitation of TCR and CD8 in a manner dependent on the membrane proximal TCRα-connecting peptide motif (α-cpm) [6;7]. This suggests a direct or indirect link through CD8β cytoplasmic regions to CD3δ and TCR [8]. However, there are several problems with these studies. The cytoplasmic region and more precisely the palmitoylation site of CD8β account for virtually all the ability of CD8 to associate with CD3-TCR [8]. Thus the observed association between CD8αβ and CD3δ-TCR may be due to co-segregation into lipid rafts. In agreement, CD3δ-CD8 interaction was significantly disrupted by *n*-octylglucoside [3], which disrupts lipid rafts [9].

Association of CD8 and TCR may be indirect, and driven by binding of small proportions of activated lck, CD3ζ and ZAP-70. Indeed several studies have shown CD8 and TCR may associate through intracellular signaling proteins [10;10-15], rather than a direct TCR-CD8 interaction. Similarly, in CD4+ T cells small proportions of TCR and CD4 are pre-assembled with activated lck and ZAP-70 in some lipid rafts [16]. In studies suggesting a direct interaction between CD8 and TCR in resting cells, free cysteines and cations were necessary for CD8-CD3δ interaction. As the binding of lck to CD8 requires free cysteines and cations [17], the observed association of CD8 and CD3δ may occur indirectly through lck [3]. Thus, CD8, CD3 and TCR may indirectly associate through larger intracellular complexes.

8.2. CD3δ and Other Receptors That Have Motifs Resembling the TCRα-cpm May Associate with CD8 on NK Cells, Dendritic Cells and Monocytes

CD8β associates with CD3δ and αβ but not γδ TCR [3]. A complex of CD8, CD3δ and TCR can be immunoprecipitated but whether any direct interaction links these proteins is not clear. If the association of TCR and CD8 is driven by formation of small percentages of activated intracellular signaling complexes [10;10-15] rather than a direct interaction of CD8 with CD3-TCR, CD8 may associate with and activate many receptors that use signaling pathways involving all or some of ZAP-70/Syk, CD3ξ/common γ chain, LAT, and lck, such as CD16 on NK cells [18-22], or others discussed above (Table 4).

On the other hand, some evidence suggests an association of CD8 with CD3δ or CD3ζz and innate immune receptors could be possible, even in monocytes. CD3δ is considered a T cell specific protein, at least in mice [23]. However, this may not be the case in humans or in particular pathological circumstances. The human monocyte cell line U937 expresses CD3δ, and expression of CD3δ increases after HIV-1 infection of these cells [24]. Furthermore, a CD3δ/ε specific mAb binds to human monocytes and macrophages, at least under some circumstances [25;26]. I have also observed binding of anti-CD3δ/ε mAb to human monocytes at low levels (data not shown). Interestingly, mouse CD8α+ dendritic cells express mRNA for CD3ε [27] and human NK cells that are CD3+ are usually CD8+ [28]. CD8β was observed to promote CD8 association to CD3δ and TCR [3]. Expression of CD8β is not limited to αβ TCR T cells, CD8β is found on macrophages [29], monocytes [30], mast cells [31] at least in rat, and γδ T cells [32] in several species. Thus, low levels of CD8 expressed on macrophages may associate with low levels of CD3δ and undefined macrophage receptors.

The α-cpm region of TCR appears important for TCR-CD8 association [8], suggesting this may be the region of TCR that interacts with CD3δ or CD8. Naeher et al. [8] suggested that the TCR α-cpm sequence FEDTxNLN that appears to mediate TCR-CD8 association (though perhaps indirectly) is not found in other proteins. However, elimination of parts of the sequence still allows TCR-CD8 association, albeit somewhat attenuated [8]. BLAST search of the TCR α-cpm sequence FEDTxNLN reveals partial conservation by other cell surface receptors (Figure A2-1, Table 1-3), such as CD36 (expressed by monocytes), Jaw1 related, CD45 and CRTAM (Class I-Restricted T cell-Associated Molecule, expressed by NK cells and T cells). These TCRα-cpm homologous sequences may or may not be accessible to CD8 or CD3δ interaction, however several pieces of evidence suggest a relationship between CD8 and some of these receptors. CD45 and CD8 both interact with lck during T cell signaling and co-immunoprecipitate [2;33-35]. CRTAM also mediates T and NK cell cytotoxicity [36], and is one of few receptors with a V-C1-set immunoglobulin domain proposed to be ancestral to the

TCR [37]. Although CD8α+ and CD8α- dendritic cells express similar levels of most cell surface antigens, CD8α+ dendritic cells express much more CD36 [38]. It will be necessary to define the sequences and structures which CD8 interacts with in CD3δ and/or TCR to determine whether CD8 may also interact with a similar sequence in other receptors.

The association of CD8 on the cell surface with other receptors may encourage partnered enhancement of complementary intracellular signaling cascades. An association of CD8 with two other cell surface receptors, namely MHC class I and CD81 has been described. The weak evidence that CD8 may co-activate cells with these receptors will be raised below.

8.3. Signaling Induced by Ligation of MHC Class I May be Enhanced by CD8

CD8 may associate with MHC class I in the plasma membrane of the same cell. This interaction has been detected by co-immunoprecipitation [39-42]. Ligation of MHC class I and MHC class II activates Syk and src kinases, as well as PKC and PI3K [43-46], akin to CD8 signaling. When MHC class I on the surface of these macrophages or dendritic cells is bound by a receptor such as TCR, CD8 may augment responses of the MHC class I-expressing cell (see Table 4). In this scenario, antigen presenting cells recognized by antigen-specific T cells, NK cells, or monocytes expressing receptors for MHC class I may activate or induce differentiation of the CD8+ antigen-presenting cell. In this way the first interaction of an antigen presenting cell with an antigen-specific T cell may modulate its ability to activate T cells subsequently.

8.4. CD8 May Associate with the Co-stimulatory Tetraspanin CD81

CD8 or CD4 immunoprecipitates with CD81 and CD82 [47]. While this interaction has not been studied for CD8, the cytoplasmic domain of CD4 is required for its association with

CD81 and CD82 [48]. CD81 is expressed by most types of human blood cells including monocytes [49]. While CD81 promotes T cell responses [50] it has unknown effects on monocyte activation and inhibits NK cell activation[51]. Some responses initiated by CD81 or CD8 are inhibited by cyclosporin A [50;52;53] and induce ERK phosphorylation [54;55]. Therefore, it is possible that CD8 and CD81 associate, and initiate intracellular signaling together. Interestingly, if CD8 and CD81 associate, CD81 could foster CD8 inclusion in lipid rafts [56], if CD8 and CD81 associate. CD81 is found on vesicles including endosomes and exosomes, and potentially contributes to intracellular trafficking towards antigen presentation [57;58]. If CD8 associates with CD81 on antigen-presenting cells, it may guide antigen-processing, and help explain the specialization of CD8α+ dendritic cells to presenting antigens from MHC class I-expressing sources such as apoptotic or virally infected cells [59;60].

Despite this apparent logic, CD8 association with a receptor that can activate monocytes or NK cells has not been previously examined. Data in this thesis demonstrate that CD8 can co-operatively signal with FcR. Given that without binding to MHC class I, CD8 is recruited to activated TCR in a manner dependent on intracellular signaling complexes [11;12;15], CD8 may be recruited to FcR and enhance FcR-initiated responses.

8.5. Figure and Figure Legend

TCRαcpm	100	FETDXNLN	108
CD36	279	FESDVNL	285
Jaw1-related isoform a	100	ETDKNL	105
CD45RC	915	ETEVNL	920
CRTAM	170	FETD _	173

Figure A2-1. Significant parts of a motif important for TCRα association with CD3δ and ζ chains, and possibly CD8 is also found in several other monocyte, NK cell and T cell receptors. Amino acids shown from TCR α connecting peptide motif (cpm) are those conserved through chicken, rat, mouse and human according to Backstrom, BT *et al. Immunity*, 5: 437. Amino acids underlined in red were demonstrated to significantly inhibit the efficiency of T cell activation (Backstrom, BT *et al. Immunity*, 5: 437). Accession numbers of other proteins above are: CD36 (NP_000063), Jaw1-related (NP_006060.2), CD45RC (NP_002829.2), CRTAM (AAH70266.1)

8.6. Literature Cited

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